

Europäisches Patentamt

European Patent Office

Office européen des brevets



(11) Publication number : **0 476 983 A1**

(12)

EUROPEAN PATENT APPLICATION

(21) Application number : 91308489.3

(51) Int. Cl.⁵ : **C07K 15/06, C07K 3/28,
C12N 15/19, C12P 21/00,
A61K 37/42**

(22) Date of filing : 18.09.91

(30) Priority : 21.09.90 US 586638
21.09.90 US 586640

(43) Date of publication of application :
25.03.92 Bulletin 92/13

(34) Designated Contracting States :
CH DE FR GB IT LI NL

(71) Applicant : **MERCK & CO. INC.**
126, East Lincoln Avenue P.O. Box 2000
Rahway New Jersey 07065-0900 (US)

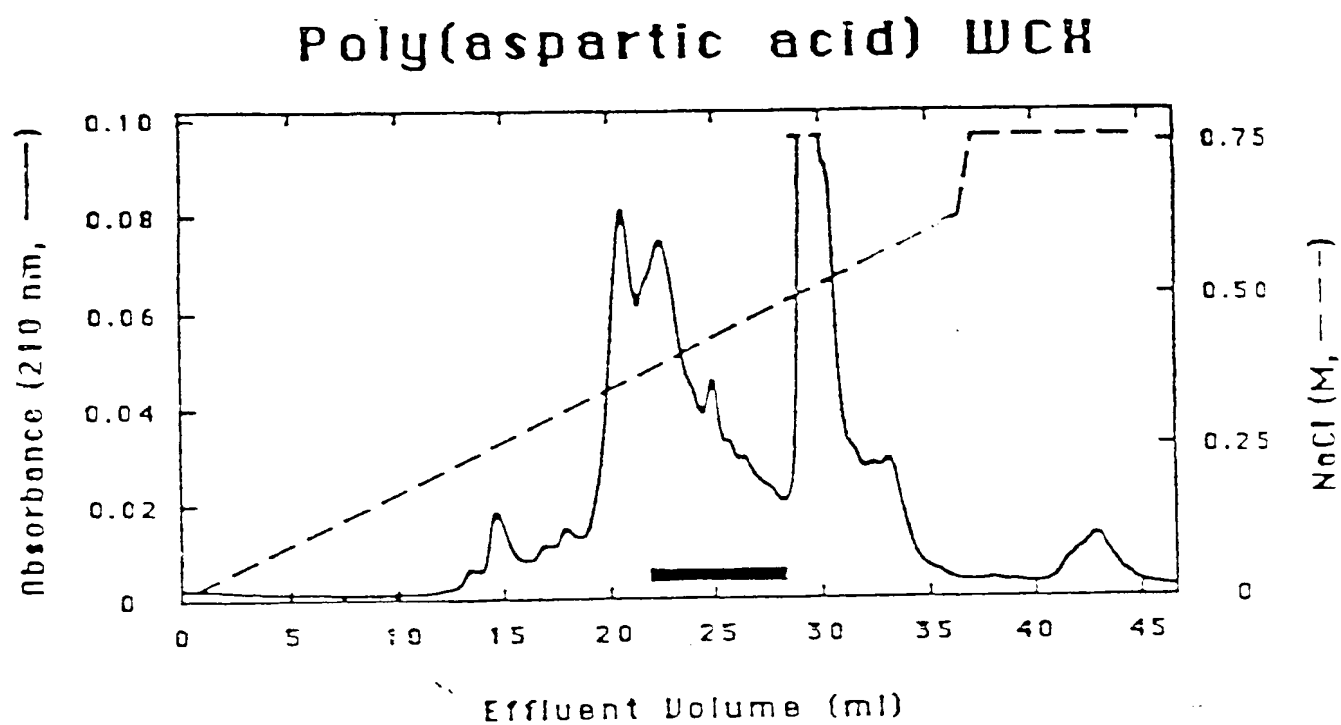
(72) Inventor : **Bayne, Marvin L.**
131 Tudor Oval
Westfield, NJ 07090 (US)
Inventor : **Conn, Gregory L.**
1751 Essex Street
Rahway, NJ 07065 (US)
Inventor : **Thomas, Kenneth A., Jr.**
245 Washington Avenue
Chatham Burrough, NJ 07928 (US)

(74) Representative : **Thompson, John Dr. et al**
Merck & Co., Inc. European Patent
Department Terlings Park Eastwick Road
Harlow, Essex CM20 2QR (GB)

(54) Vascular endothelial cell growth factor II.

(57) Vascular endothelial cell growth factor II is purified from the culture media used to maintain mammalian glioma cells. The protein is a heterodimer, stimulates mitogenesis of mammalian vascular endothelial cells and is useful for the promotion of vascular development and repair. This unique growth factor is also useful in the promotion of tissue repair.

FIG. 1



BRIEF DESCRIPTION OF THE DRAWING

Figure 1. VEGF II activity present in fractions eluting from a polyaspartic acid WCX HPLC cation exchange column, bar denotes pooled active fractions.

Figure 2. VEGF II activity present in fractions eluting from a metal chelate column.

Figure 3. VEGF II activity present in fractions eluting from a RP-HPLC C₄ column.

Figure 4. Full length amino acid residue protein translation product and its cDNA coding sequence for VEGF I A subunit plus polypeptide cleavage products used to determine the amino acid sequence.

Figure 5. Full length amino acid residue protein translation product and its cDNA coding sequence for VEGF II A subunit plus polypeptide cleavage products used to determine the amino acid sequence.

Figure 6. Full length amino acid residue protein translation product and its cDNA coding sequence for the 135 amino acid form of VEGF II B subunit plus polypeptide cleavage products used to determine the amino acid sequence.

Figure 7. Full length amino acid residue protein translation product and its cDNA coding sequence for the 115 amino acid form of VEGF II B subunit.

BACKGROUND OF THE INVENTION

A new class of cell-derived dimeric mitogens with apparently restricted specificity for vascular endothelial cells has recently been identified and generally designated vascular endothelial growth factor (VEGF). The mitogen has been purified from: conditioned growth media of rat glioma cells, [Conn et al., Proc. Natl. Acad. Sci. USA 87: 2628-2632 (1990)]; conditioned growth media of bovine pituitary folliculo stellate cells [Ferrara and Henzel, Biochem. Biophys. Res. Comm. 161: 851-858 (1989) and Gospodarowicz et al., Proc. Natl. Acad. Sci. USA 86: 7311-7315 (1989)]. Vascular endothelial growth factor I (VEGF I) is a homodimer with an apparent molecular mass of 46 kDa, with each subunit having an apparent molecular mass of 23 kDa. VEGF I has distinct structural similarities to platelet-derived growth factor (PDGF), a mitogen for connective tissue cells but not vascular endothelial cells from large vessels.

OBJECTS OF THE INVENTION

It is, accordingly, an object of the present invention to provide a novel vascular endothelial growth factor II (VEGF II) free of other proteins. Another object is to provide a procedure for the purification of the substantially pure VEGF II. A further object is to provide VEGF II to stimulate endothelial cells for induction of blood vessel growth, vascular repair and the production of artificial blood vessels. Another object is to provide VEGF II to stimulate tissue repair.

SUMMARY OF THE INVENTION

Vascular endothelial cell growth factor II is purified from the culture media used to maintain mammalian glioma cells. The protein is a heterodimer and stimulates mitogenesis of mammalian vascular endothelial cells and is useful for the promotion of vascular development and repair. This unique growth factor is also useful in the promotion of tissue repair.

DETAILED DESCRIPTION

The present invention relates to a unique vascular endothelial cell growth factor (designated VEGF II), isolated and purified from glioma cell conditioned medium, which exhibits mitogenic stimulation of vascular endothelial cells. Glioma is defined herein as any neoplasm derived from one of the various types of cells that form the interstitial tissue of the central nervous system including brain, spinal cord, posterior pituitary gland and retina. Consequently, the scope of the present invention is intended to include the unique growth factor isolated and purified from any mammalian glioma tissue or other cells including cell lines. Cell lines include, but are not limited to, glioma-derived cell lines such as C6, hs 683 and GS-9L; glioblastomas such as A-172 and T98G; neuroblastomas such as IMR-32 and SK-N-MC; neurogliomas such as H4; teratomas such as XB-2; astrocytomas such as U-87 MG and U-373 MG; embryonal carcinomas and non-transformed glial or astrocyte cell lines, and the human medulloblastoma line TE 671, with GS-9L and TE 671 being preferred. VEGF II is present and can be isolated from rat tissue including ovary, heart and kidney. Anterior pituitary tumor cell lines such as GH3 and Hs 139 may also be used. Although the VEGF of this invention is described as being isolated from glioma conditioned medium, it may be isolated from any mammalian conditioned medium.

Glioma cells such as the rat cell line GS-9L are grown to confluence in tissue culture flasks, about 175 cm², in a cell culture medium such as Dulbecco's Modified Eagle's Medium (DMEM) supplemented with about 10% newborn calf serum (NCS). When the cells reach confluence the culture medium is removed, the cell layers are washed with Ca²⁺/Mg²⁺-free phosphate buffered saline (PBS) and are removed from the flasks by treatment with a solution of trypsin, about 0.1%, and EDTA, about 0.04%. The cells, about 1×10^3 , are pelleted by centrifugation, resuspended in about 1500 ml of DMEM containing about 5% NCS and plated into a ten level cell factory (NUNC) 6,000 cm² surface area. The cells are incubated for about 48 to about 96 hours, with 70 hours preferred, at about 37°C in an atmosphere of about 5% CO₂. Following incubation the medium is removed and the cell factories are washed about 3 times with PBS. About 1500 ml of fresh culture media is added containing about a 1:2 mixture of Ham's F12/DMEM containing about 15 mM HEPES, about 5 µg/ml insulin, about 10 µg/ml transferrin and with or without about 1.0 mg/ml dexamethasone serum (dexam). This media is replaced with fresh media after about 24 hr and collected every 48 hr thereafter. The collected conditioned media is filtered through Whatman #1 paper to remove cell debris and stored at about -20°C.

[illegible]

and 28.5 ml see Fig. 1.

The active fractions eluted from the polyaspartic WCX column that contain VEGF II are pooled, adjusted to about pH 7.0 and loaded onto a 1 x 10 cm column of Pharmacia Chelating Sepharose 6B charged with an excess of copper chloride and equilibrated in about 0.05 M sodium phosphate, about pH 7.0, containing about 2 M NaCl and about 0.5 mM imidazole (A buffer). VEGF II is eluted from the column with a gradient from 0-20% B over 10 minutes, 20-35% B over 45 minutes and 35-100% B over 5 minutes at a flow rate of 0.3 ml/min, where B buffer is 0.05 M sodium phosphate, pH 7.0, containing about 2 M NaCl and 100 mM imidazole. The active fractions containing VEGF II activity eluted between about 12.6 and 22.8 ml of the gradient effluent volume, see Fig. 2.

The pooled fractions containing VEGF II activity eluted from the metal chelate column are loaded onto a 4.6 mm x 5 cm Vydac C₄ reverse phase HPLC column (5 µm particle size) previously equilibrated in solvent A [0.1% trifluoroacetic acid (TFA)]. The column is eluted with a linear gradient of about 0 to 30% solvent B over 15 minutes, 30% B for an additional 15 minutes, then 30-45% B over 22.5 minutes and finally 45-100% B over 5.5 minutes. Solvent B consists of solvent A containing 67% acetonitrile (v/v). The flow rate is maintained at about 0.75 ml/min and fractions are collected every minute. The homogeneous VEGF II elutes from the C₄ column under these conditions at between about 32 and about 38 ml of the gradient effluent volume, see Fig. 3.

Purity of the protein is determined by sodium dodecylsulfate (SDS) polyacrylamide gel electrophoresis (PAGE) in 12.5% crosslinked gels using the technique of Laemmli, Nature 227: 680-684 (1970). The silver stained gels show VEGF II to consist of one band under non-reducing conditions with an approximate apparent molecular mass of about 58,000 daltons. When a sample containing the microheterogeneous forms of VEGF II is separated under reducing conditions it migrates as two about 23 kilodalton (kDa) subunits. The purification process results in VEGF II that is essentially free of other mammalian cell products, such as proteins. Recombinantly derived VEGF II will also be free of mammalian cell products.

Biological activity is determined by mitogenic assay using mammalian vascular endothelial cells. Human umbilical vein endothelial (HUVE) cells are plated on gelatin-coated dishes at a density of about 5000 cells per well in about 500 µl of Medium 199 (M199) containing about 20% heat-inactivated fetal calf serum (FCS). Samples to be assayed are added at the time of plating. The tissue culture plates are incubated at about 37° C for about 12 hours and about 2 microcuries of tritiated thymidine (NEN, 20 Ci/mmol) is added per ml of assay medium (1.0 µCi/well). The plates are incubated for a further 60 hr, the assay medium is removed and the plates are washed with Hanks balanced salt solution containing about 20 mM Hepes, about pH 7.5, and about 0.5 mg/ml bovine serum albumin. The cells are lysed and the labelled DNA solubilized with about 200 µl of a solution containing about 2 gm of sodium carbonate and about 400 mg sodium hydroxide in about 100 ml water. The incorporated radioactivity was determined by liquid scintillation counting. The concentration of VEGF which elicited a half-maximal mitogenic response in HUVE cells was approximately 2 ± 1 ng/ml. The glycosaminoglycan heparin, which is required in these assays at a level of 10-100 µg/ml to promote a response to a positive control, acidic fibroblast growth factor, does not enhance mitogenic stimulation of these cells by VEGF II.

A purified about 1-2 µg sample of VEGF II is reduced in about 0.1 M Tris, about pH 9.5, with about 0.1% EDTA, about 6 M guanidinium chloride and about 20 mM dithiothreitol for about 2 hr at about 50° C. The reduced protein is carboxymethylated for about 1 hour in a solution containing about 9.2 µM of unlabelled and 2.8 µM of ¹⁴C-methiodiacetic acid in about 0.7 M Tris, about pH 7.8, and about 0.1% EDTA and about 6 M guanidinium chloride. The protein is carboxymethylated for about 1 hr at room temperature. The protein is isolated after reduction and carboxymethylation by reverse phase HPLC chromatography on a Vydac C₄ column, about 4.6 mm x 5 cm. The protein is loaded onto a column pre-equilibrated with about 0.1% TFA and eluted by a 45 ml linear gradient from about 0.1% TFA to 0.1% TFA/67% acetonitrile at a flow rate of about 0.75 ml/min. The reduced and carboxymethylated protein eluted as two peaks at approximately 25 and 28 ml with the proportion being approximately equal as determined by monitoring absorbance at 210 nm.

Samples of the reduced and carboxymethylated monomers are applied to polybrene-coated glass fiber filters and their N-terminal sequences are determined by Edman degradation in an ABI gas phase microsequencer in conjunction with an ABI 120A on-line phenylthiohydantoin analyzer following the manufacturers instructions. The protein showing the peak of absorbance eluting at approximately 25 ml (A subunit or monomer) yielded an amino terminal sequence of:

Asn Pro Thr Thr Gly Gly Gly Gly Lys Asn Lys Gly Val Val

which is identical to the monomers of VEGF I, Chan et al., Proc. Natl. Acad. Sci. USA 87: 2628-2632 (1990). The peak of absorbance eluting at approximately 28 ml (B subunit or monomer) yielded an N-terminal sequence of:

Asn Lys Ser Asn Gly Asn Xxx Ser Thr Gly Met Gly Val Val Pro Phe Asn Gly Val

where xxx is a small amount of a truncated form of the same sequence missing the last three amino acid residues. The monomer of VEGF II is a dimer of the B subunit. The monomer of VEGF I is a dimer of the A subunit.

single Asn occurs in a class of Asn-Xxx-Ser-Thr-N-glycosylation sequence, it is presumed to be glycosylated. The A subunit and the total of both B subunits are recovered in nearly equal amounts supporting the interpretation that the two peptides combine to form an AB heterodimer in VEGF II.

A sample of the A monomer was treated with either the protease trypsin which cleaves polypeptides on the C-terminal side of lysine and arginine residues or Lys-C which cleaves polypeptides on the C-terminal side of lysine by procedures well known in the art. The peptides are isolated by reversed phase HPLC (RP-HPLC). The amino acid sequences of the isolated peptides are determined using the Edman degradation in the ABI gas phase sequencer in conjunction with the ABI 120 A on-line phenylthiohydantoin analyzer following manufacturer's instructions. The amino acid sequences are shown in Figure 4.

Reduced and carboxymethylated A monomer is dried and solubilized in about 0.1 M Tris, about pH 7.8, about 6 M guanidinium chloride containing about 0.1% EDTA. V8 protease is added in 0.1 M ammonium bicarbonate buffer, about pH 8.0, and the mixture is incubated for about 48 hr at about 37°C. The protease cleaves predominantly on the carboxy terminal side of glutamic acid residues. The resulting polypeptides were resolved by C₁₈ RP-HPLC as above.

The reduced and carboxymethylated A subunit protein solution is adjusted to a pH of about 6.8 with 6 N HCl and dithiothreitol is added to a final concentration of 2 M for reduction of any methionine sulfoxide to methionine residues. After about 20 hr of reduction at about 36°C the protein is rebuffered by C₁₈ HPLC. The product is dried and cleaved on the carboxy terminal side of methionine residues by 200 μ l of 40 mM cyanogen bromide in about 70% (v/v) formic acid under an argon atmosphere at about 20°C for about 24 hr in the dark. The cleavage products are resolved by C₁₈ RP-HPLC. The amino acid sequences shown in Figure 4, see Conn et al., *Proc. Natl. Acad. Sci. USA* 87: 2623-2632, 1990.

The full length 164 amino acid residue protein translation product of the VEGF II A monomer, which is now known to be identical with the VEGF II A monomer and its cDNA coding sequence are shown in Fig. 4 and Fig. 5. The mature amino terminus begins at residue 27, immediately following a typical hydrophobic secretory signal sequence. A single potential N-glycosylation site exists at Asn101. Most 143 amino acid residues of the 164 residues of the reduced and carboxymethylated mature subunit including the amino terminus and HPLC reversed phase-purified products of tryptic (T), Lys-C (L), *Staphylococcus aureus* V8 protease (V8) and cyanogen bromide (CB) cleavages, were determined by direct microsequencing (Applied Biosystems 470A) using a total of 5 μ g of protein. All residues identified by amino acid sequencing are denoted by arrows pointing to the right either directly beneath the mature prosequence following the bracket at residue 27 for the amino terminal determination of the whole subunit or for residues identified from the polypeptide cleavage products above the double-headed arrows spanning the length of the particular polypeptide. One listed pair of polypeptides, V16A and V16B, was sequenced as a mixture and, therefore, are only confirmatory of the cDNA-derived amino acid sequence, see Figure 4.

Samples of the reduced and carboxymethylated pure VEGF II A and B monomers were each digested with the Lys-C endoprotease which cleaves polypeptides on the C-terminal side of lysine residues. The peptides were isolated by reverse phase HPLC and their amino acid sequences were determined as described above. The locations of the peptides in the final VEGF II A and B sequences are shown in Fig. 5 and Fig. 6, respectively.

The full length coding region of the A monomer is determined from three sets of overlapping cDNA clones. Degenerate oligonucleotide primers based on the amino acid sequences Phe-Met-Asp-Val-Tyr-Gln from polypeptide 142, residues 42-47, and Cys-Lys-Asn-Thr-Asp from polypeptide 108, residues 164-168 (see Fig. 4 and 5) were used to PCR amplify the central region of the cDNA for VEGF A chain following the procedure of Saiki et al., *Science* 232: 1329-1334, 1987. A single band migrating at 420 bp was purified, digested with *Sac*I, ligated into pGEM3Z(+) and sequenced. The nucleotide sequence obtained, p4238, was used as a template for antisense and sense PCR primers to amplify the 5' and 3' ends of the cDNA according to the protocol of Saiki et al., *Proc. Natl. Acad. Sci. USA* 85: 8898-8902, 1988. These 5' and 3' clones are designated p5-15 and p3-15, respectively. Regions of complete cDNA sequences excluding the primers, determined by direct sequencing are indicated by double-headed arrows above the nucleotide sequence. In addition to the cDNA coding the 164 amino acid secreted form, identified by protein sequencing, two alternative cDNA clones encode a 121 amino acid and a 188 amino acid form are cloned and sequenced.

The full length coding region of the B monomer is determined from four sets of overlapping cDNA clones. Degenerate oligonucleotide primers based on the amino acid sequences Phe-Met-Asp-Val-Tyr-Gln from polypeptide 142, residues 42-47, and Cys-Lys-Asn-Thr-Asp from polypeptide 108, residues 164-168 (see Fig. 4 and 5) were used to PCR amplify the central region of the cDNA for VEGF B chain following the procedure of Saiki et al., *Science* 232: 1329-1334, 1987. A single band migrating at 1.8 kb was purified, digested with *Sac*I, ligated into pGEM3Z(+) and sequenced. The nucleotide sequence obtained, p1800, was used as a template for antisense and sense PCR primers to amplify the 5' and 3' ends of the cDNA according to the protocol of Saiki et al., *Proc. Natl. Acad. Sci. USA* 85: 8898-8902, 1988. These 5' and 3' clones are designated p5-18 and p3-18, respectively. Regions of complete cDNA sequences excluding the primers, determined by direct sequencing are indicated by double-headed arrows above the nucleotide sequence. In addition to the cDNA coding the 164 amino acid secreted form, identified by protein sequencing, two alternative cDNA clones encode a 121 amino acid and a 188 amino acid form are cloned and sequenced.

from GGAGTGCATAT RNA. Regions of complete DNA sequences (excluding the primers) determined for each set of clones are indicated by double-headed arrows above the nucleic acid sequence. The entire base sequence for the 135 amino acid monoheterogeneous B subunit and the 115 amino acid monoheterogeneous B subunit are shown in Fig. 6 and Fig. 7.

It is intended that vascular endothelial cell growth factor II exist as a heterodimer consisting of an A subunit and a B subunit. It is further intended that VEGF homodimers exist as either two A subunits or two B subunits. The B subunit may be either the 135 amino acid form or the 115 amino acid form. The A subunit of the may be either the 169 amino acid form, the 164 amino acid form or the 120 amino acid form. The heterodimers or homodimer species can be depicted as: $A_{169} + B_{135}$, $A_{169} + B_{115}$, $A_{164} + B_{135}$, $A_{164} + B_{115}$, $A_{120} + B_{135}$, $A_{120} + B_{115}$, $B_{135} + B_{135}$, $B_{135} + A_{169}$, $A_{169} + A_{169}$ and $A_{169} + A_{120}$. The homodimers can be depicted as: $B_{135} + B_{135}$, $B_{115} + B_{115}$, $A_{169} + A_{169}$, and $A_{120} + A_{120}$. It is also intended that the invention include all of the individual subunit forms of both the A subunit and the B subunit of VEGF II.

It is further intended that the nucleotide sequence for vascular endothelial growth factor II be interpreted to include all codons that code for the appropriate amino acids in the sequence for each of the vascular endothelial growth factor II subunits, as indicated by the degeneracy of the genetic code. It is further intended that the nucleotide sequence and the amino acid sequence for VEGF II subunits include truncated genes or proteins which result in a protein which exhibits biological activity similar to vascular endothelial growth factor II. The scope of the invention is intended to include all naturally occurring mutations and allelic variants and any randomly generated artificial mutants which may change the sequences but do not alter biological activity as determined by the ability to stimulate the division of vascular endothelial cells.

The above described heterodimers, homodimers, subunits and monomers of vascular endothelial growth factor II are characterized by being the product of chemical synthetic procedures or of prokaryotic or eukaryotic

expression of the DNA sequences as described herein. A monomer is defined as a subunit that cannot form a dimeric unit. Expression of the recombinant VEGF II genes (recombinant DNA) is accomplished by

a number of different host cells which contain at least one of a number of expression vectors. Expression vectors

is defined herein as DNA sequences that are required for the transcription of cloned copies of recombinant

DNA sequences or genes and the translation of their mRNAs in an appropriate host. Such vectors can be used to express genes in a variety of hosts such as bacterial, blue-green algae, yeast cells, insect cells, plant cells

and animal cells, with mammalian cells being preferred. The genes may also be expressed in a number of virus

systems. Specifically designated vectors allow the shuttling of DNA between bacteria-yeast, bacteria-plant or

bacteria-animal cells. An appropriately constructed expression vector should contain: an origin of replication for autonomous replication in host cells; selective markers; a limited number of useful restriction enzyme sites;

an phage number; and strong promoters. A promoter is defined as a DNA sequence that directs RNA polymerase to bind to DNA and to initiate RNA synthesis. A strong promoter is one which causes mRNAs to be initiated

with high frequency. Expression vectors may include, but are not limited to, cloning vectors, modified cloning vectors, specially designed plasmids or viruses and cosmids. The expression of mammalian genes in cultured

mammalian cells is well known in the art. Sambrook et al., Molecular Cloning: A Laboratory Manual, 2nd Edition, Book 8, Cold Spring Harbor Laboratory Press, 1989, and Current Protocols in Molecular Biology, Ausubel et al., Eds., Greene Publishing Associates and Wiley-Interscience, 1987, and supplements, disclose various mam-

malian expression vectors and vector systems along with methods for the introduction of recombinant vectors

into mammalian cells. The cDNA for the monomer forms of the A and B subunits can be expressed in a system such as that described by Linemeyer et al., European Patent Application Publication No. 269,958. The cDNA

is incorporated into a commercial available plasmid such as pRII 229-3 (Pharmacia) as modified as by Linemeyer et al., and expressed in E. coli. Other expression systems and host cells are well known in the art.

The high DNA content and glycosylation of the A and B subunits along with the structure of the homodimeric and heterodimeric suggests that expression of biologically active proteins is carried out in animal cells. Expression

may be carried out in Chinese hamster ovary (CHO) cells with the cloned VEGF II DNA cotransfected with the gene for neomycin resistance (neo^r) into neo^r CHO cells (see Sambrook et al.). Transformants express

and are selected on media lacking neomycin and are exposed to increasing concentrations of methanol. The anti-VEGF II genes are thus amplified, leading to a stable cell line capable of expressing

the cloned VEGF II. The plasmid is designed to include either an A subunit and a B subunit or two A or B subunits. The two cDNAs are operatively situated so that the protein produced will be a dimer and will have VEGF

biological activity. In contrast with the heterodimer, an appropriate nucleic acid arrangement of nucleotide sequence cDNA subunits may give rise to the desired protein which is produced by cells containing an expression

vector containing the appropriate cloned gene of DNA sequence in a suitable

a B subunit. Expression vectors capable of expressing homodimeric forms of VEGF will contain either one or two DNA sequences which encode either two A or two B subunits.

The ability of the various species of VEGF II to stimulate the division of vascular endothelial cells makes this protein in all microheterogeneous forms and alternative splicing forms useful as a pharmaceutical agent. The protein as used herein is intended to include all microheterogeneous forms as previously described. The protein can be used to treat wounds of mammals including humans by the administration of the novel protein to patients in need of such treatment.

The novel method for the stimulation of vascular endothelial cells comprises treating a sample of the desired vascular endothelial cells in a nutrient medium with mammalian VEGF II, preferably human or rat, at a concentration of about 1-10 ng/ml. If the vascular endothelial cell growth is conducted *in vitro*, the process requires the presence of a nutrient medium such as DMEM or a modification thereof and a low concentration of calf or bovine serum such as about 0 to 2% by volume. Preservatives such as antibiotics may also be included; these are well known in the art.

The novel growth factors of this invention are useful for the coverage of artificial blood vessels with vascular endothelial cells. Vascular endothelial cells from the patient would be obtained by removal of a small segment of peripheral blood vessel or capillary-containing tissue and the desired cells would be grown in culture in the presence of VEGF II and any other supplemental components that might be required for growth. After growth of adequate numbers of endothelial cells in culture to cover a synthetic polymeric blood vessel the cells would be plated on the inside surface of the vessel, such as fixed umbilical vein, which is then implanted in the patient. Alternatively, tubular supports are coated *in vitro* with VEGF II prior to implantation into a patient. Following implantation endothelial cells migrate into and grow on the artificial surface. Prior coating of the artificial vessel either covalently or noncovalently, with proteins such as fibrin, collagen, fibronectin or laminin would be performed to enhance attachment of the cells to the artificial surface. The cell-lined artificial vessel would then be surgically implanted into the patient and, being lined with the patient's own cells, would be immunologically compatible. The non-thrombogenic endothelial cell lining should decrease the incidence of clot formation on the surface of the artificial vessel and thereby decrease the tendency of vessel blockage or embolism elsewhere.

The novel proteins are also useful for the production of artificial vessels. Vascular endothelial cells and smooth muscle cells from the patient would be obtained and grown separately in culture. The endothelial cells would be grown in the presence of VEGF II as outlined above. The smooth muscle would be grown in culture by procedures well known in the art. A tubular mesh matrix of a biocompatible polymer (either a synthetic polymer, with or without a coating of proteins, or a non-immunogenic biopolymeric material such as surgical suture thread) would be used to support the culture growth of the smooth muscle cells on the exterior side and vascular endothelial cells on the interior surface. Once the endothelial cells form a confluent monolayer on the inside surface and multiple layers of smooth muscle cells cover the outside, the vessel is implanted into the patient.

The novel peptides can also be used for the induction of tissue repair or growth. The pure VEGF II would be used to induce and promote growth of tissue by inducing vascular growth and/or repair. The peptide can be used either topically for tissue repair or intravascularly for vascular repair. For applications involving neovascularization and healing of surface wounds the formulation would be applied directly at a rate of about 10 ng to about 1 mg/cm²/day. For vascular repair VEGF II is given intravenously at a rate of about 1 µg to about 100 µg/kg/day of body weight. For internal vascular growth, the formulation would be released directly into the region to be neovascularized either from implanted slow release polymeric material or from slow release pumps or repeated injections. The release rate in either case is about 100 ng to about 100 µg/day/cm².

For non-topical application the VEGF is administered in combination with pharmaceutically acceptable carriers or diluents such as, phosphate buffer, saline, phosphate buffered saline, Ringer's solution, and the like, in a pharmaceutical composition, according to standard pharmaceutical practice. For topical application, various pharmaceutical formulations are useful for the administration of the active compound of this invention. Such formulations include, but are not limited to, the following: ointments such as hydrophilic petrolatum or polyethylene glycol ointment; pastes which may contain gums such as xanthan gum; solutions such as alcoholic or aqueous solutions; gels such as aluminum hydroxide or sodium alginate gels; albumins such as human or animal albumins; collagens such as human or animal collagens; celluloses such as alkyl celluloses, hydroxy alkyl celluloses and alkylhydroxyalkyl celluloses, for example methylcellulose, hydroxyethyl cellulose, carboxymethyl cellulose, hydroxypropyl methylcellulose, and hydroxypropyl cellulose; polyoxamers such as Pluronic® Polymers exemplified by Pluronic® F-127, tetronics such as tetronic 1508, and alginates such as sodium alginate.

The following examples illustrate the present invention without, however, limiting the same thereto.

EXAMPLE 1Preparation of Medium Conditioned By GS-9L Cells

GS-9L cells were grown to confluence in 175 cm² tissue culture flasks in Dulbecco's Modified Eagle's Medium/10% newborn calf serum (DMEM/CS). At confluence the medium was decanted from the flasks, the flasks were washed with calcium and magnesium free phosphate buffered saline (PBS) and the cells were removed by treatment with a 1X solution of trypsin/EDTA. The cells (1×10^6) were pelleted by centrifugation, resuspended in 1500 ml of DMEM/5% NCS and plated into a ten level (6000 cm² surface area) cell factory (NUNC). After 72 hours incubation at 37° C in a 5% CO₂ atmosphere the medium was decanted and the cell factories were washed 3 times with PBS. The cells were refed with 1500 ml of a 1:2 mixture of Ham's F-12/DMEM containing 25 mM Hepes, 5 μ g/ml insulin, 10 μ g/ml transferrin and 1.0 mg/ml bovine serum albumin. This medium was changed with fresh F-12/DMEM after 24 hours and collected every 48 hours after that. The conditioned medium was filtered through a Whatman #1 paper to remove cell debris and stored frozen at -20°C.

EXAMPLE 2Carboxymethyl-Sephadex Chromatography

GS-9L conditioned medium, from Example 1, was thawed and brought to pH 6.0 with 1 M HCl. Two grams CM Sephadex C-50 cation exchange (Pharmacia) resin pre-equilibrated in PBS adjusted to pH 6.0 with 1 N HCl was added to 20 liters of conditioned medium. The mixture was stirred at low speed for 24 hours at 4° C. The resin was then allowed to settle and the medium is siphoned off. The remaining resin slurry was packed into a 3.0 cm diameter column and any remaining medium is allowed to drain off. Unbound protein was washed off the column with 0.05 M sodium phosphate, pH 6.0, containing 0.15 M NaCl. Vascular endothelial growth factor activity was eluted from the column with a subsequent wash of 0.05 M sodium phosphate, pH 6.0, containing 0.6 M NaCl.

EXAMPLE 3Concanavalin A Con A Lectin Affinity Chromatography

A 3.9 cm diameter column containing about 5 ml of packed Con A agarose (Vector Laboratories) was equilibrated with 0.05 M sodium acetate, pH 6.0, containing 1 mM CaCl₂, 1 mM MgCl₂ and 0.6 M NaCl. The active eluate from the CM Sephadex C-50 column, Example 2, was applied to the Con A agarose and unbound protein was washed from the column with equilibration buffer. The column was then rinsed with three column volumes of 0.05 M sodium acetate, pH 6.0, containing 1 mM CaCl₂, 1 mM MgCl₂ and 0.1 M NaCl. Bound protein was subsequently eluted from the column by application of this buffer supplemented with 0.33 M α -methyl mannopyranoside and 0.28 M α -methyl glucoside.

EXAMPLE 4Aspartic Acid WAX HPLC Cation Exchange Chromatography

The active eluate from the Con A column, Example 3, was applied to a 25 cm x 4.6 mm poly(aspartic acid) WAX cation exchange HPLC column (Nest Group) pre-equilibrated in 0.05 M sodium phosphate buffer, pH 6.0. The column was eluted with a linear gradient of 0 to 0.75 M NaCl in this buffer over 60 minutes at a flow rate of 1.5 ml/min collecting 0.75 ml fractions. VEGF II activity present in fractions eluting between approximately 21.7 and 28.5 min. were pooled as shown by solid horizontal bar in Fig. 1.

EXAMPLE 5Octyl Sulfate Chromatography

The active fraction eluted from the poly(aspartic acid) WAX column, Example 4, that contains VEGF II were adjusted to pH 7.0 and loaded onto a 1 x 10 cm column of Pharmacia Cheating Sepharose 6B charged with octyl sulfate (octylsulfate) and equilibrated in 0.05 M sodium phosphate, pH 7.0, containing 0.1 M NaCl. The column was then washed with a buffer. VEGF II was eluted from the column with a gradient from 0.05% B over 10

minutes, 20-35% B over 45 minutes and 35-100% B over 5 minutes at a flow rate of 0.3 ml/min, where B buffer was 0.05 M sodium phosphate, pH 7.0, containing 2 M NaCl and 100 mM imidazole. The active fractions containing VEGF II activity eluting between 12.6 and 22.8 ml of the gradient effluent volume were pooled as shown by the solid horizontal bar in Fig. 2.

EXAMPLE 6

Reverse Phase Chromatography

The fractions containing VEGF II activity pooled from the metal chelate column, Example 5 were loaded onto a 4.6 mm x 5 cm Vydac C4 reverse phase HPLC column (5 μ m particle size) equilibrated in solvent A (0.1% trifluoroacetic acid (TFA)). The column was eluted with a gradient of 0-30% solvent B over 15 minutes, 30% B for an additional 15 minutes, then 30-45% B over 22.5 minutes and finally 45-100% B over 5.5 minutes where solvent B = A containing 67% acetonitrile. The flow rate was maintained at 0.75 ml/min. The active VEGF II fractions eluting between approximately 32.2 and 37.5 ml of the gradient effluent volume were pooled as shown by the solid horizontal bar in Fig. 3.

EXAMPLE 7

Mitogenic Assays

Human umbilical vein endothelial cells (HUVE) were plated on gelatin-coated 48 well tissue culture dishes at a density of 5000 cells/well in 500 μ l of Medium 199 containing 20% heat inactivated fetal calf serum (FCS). Samples to be assayed were added at the time of plating. The tissue culture plates are incubated at 37°C for 12 hours and 2 microcuries of tritiated thymidine (NEN, 20 Ci/mmol) was added per ml of assay medium (1.0 μ Ci/well). The plates were incubated for a further 60 hr, the assay medium was removed and the plates were washed with Hanks balanced salt solution containing 20 mM Hepes, pH 7.5, and 0.5 mg/ml bovine serum albumin. The cells were lysed and the labelled DNA solubilized with 200 μ l of a solution containing 2 gm of sodium carbonate and 400 mg sodium hydroxide in 100 ml water. The incorporated radioactivity was determined by liquid scintillation counting.

The concentration of VEGF II which elicited a half-maximal mitogenic response in HUVE cells was approximately 2 ± 1 ng/ml. The glycosaminoglycan heparin, which is required in these assays at a level of 10-100 μ g/ml to promote a response to a positive control, acidic fibroblast growth factor, does not enhance mitogenic stimulation of these cells by VEGF II.

EXAMPLE 8

Purity And Protein Structure Characterization of VEGF II

Purity of the protein under non-reducing conditions was determined by SDS-PAGE in 12.5% crosslinked gels according to the method of Laemmli, Nature 227: 680-685 (1970). The silver-stained gel contained a single band with an apparent mass of approximately 58 kDa. VEGF II migrated in SDS-PAGE under reducing conditions in 15% crosslinked gels as a broad silver-stained band with apparent molecular mass of approximately 23 kDa.

VEGF II was stored at 4°C in the aqueous trifluoroacetic acid (TFA)/acetonitrile mixture used to elute the homogeneous protein in reversed phase C4 HPLC chromatography at the final stage of the purification protocol previously described. Aliquots of the purified protein (1-2 μ g) were vacuum evaporated to dryness in acid-washed 10 x 75 mm glass tubes and reduced for 2 hours at 50°C in 100 μ l of 0.1 M Tris buffer, pH 9.5, and 6 M guanidinium chloride containing 0.1% EDTA and 20 mM dithiothreitol (Calbiochem, Ultrol grade) under an argon atmosphere. The reduced protein was subsequently carboxymethylated for 1 hour at 20°C by the addition of 100 μ l of 0.7 M Tris, pH 7.8, containing 0.1% EDTA, 6 M guanidinium chloride, 9.2 μ M unlabeled iodoacetic acid and 50 μ Ci of iodo[2- 14 C]acetic acid (17.0 mCi/mmol, Amersham). After completion of the carboxymethylation, the mixture was loaded directly onto a 4.6 mm x 5.0 cm Vydac C4 column which had been pre-equilibrated in 0.1% TFA. The reduced and carboxymethylated protein was repurified by elution with a 45 minute linear gradient of 0 to 67% (v/v) acetonitrile in 0.1% TFA at a flow rate of 0.75 ml/min and stored in this elution solution at 4°C. The reduced and carboxymethylated protein eluted as two peaks at approximately 25 and 28 ml that were of approximately equal area as determined by monitoring absorbance at 210 nm.

Reduced and carboxymethylated protein and non-reduced and non-carboxymethylated protein were each applied to

polybrene-coated glass fiber filters and their N-terminal sequences were determined by Edman degradation in an ABI gas phase microsequencer in conjunction with an ABI 120A on-line phenylthiohydantoin analyzer following manufacturers instructions. The peak of absorbance eluting at approximately 28 ml (A subunit) yielded an amino terminal sequence APTEEGEDKAHEVM identical to VEGF₁. The peak of absorbance eluting at approximately 26 ml (B subunit) yielded the N-terminal sequence ALSAGN(K)STEMEVVPNEV plus a nearly equal amount of a truncated form of the same sequence missing the first three residues. The missing K residue corresponds to an Asn in the cloned sequence. Since this missing Asn occurs in a classical Asn-X-Ser/Thr N-glycosylation sequence it is presumed to be glycosylated. The A and sum of the B chain peptides were recovered in nearly equal amounts supporting the interpretation that the two peptides combine to form an AB heterodimer in VEGF II.

Reduced and carboxymethylated A and B subunits (650 ng each) were each dried by vacuum evaporation in acid-washed 10 x 75 mm glass tubes. Lys C protease (50 ng, Boehringer Mannheim), an enzyme that cleaves on the carboxyl terminal side of lysine residues, was added to each tube in 100 μ l of 25 mM Tris, pH 8.5, 0.1% EDTA. The substrate protein subunits were separately digested at 37°C for 8 hours and the resulting polypeptides resolved by reversed phase HPLC chromatography on a 4.6 mm x 25 cm Vydac C₁₈ column equilibrated in 0.1% TFA. Polypeptides were fractionated by elution with a 2 hour linear gradient of 0-67% acetonitrile in 0.1% TFA at a flow rate of 0.75 ml/min at 20°C. Individual peaks were manually collected and stored in this elution solution at 4°C.

The amino acid sequences of the isolated peptides were then determined using Edman degradation in an ABI gas phase sequencer in conjunction with the ABI 120A on-line phenylthiohydantoin analyzer (Applied Biosystems Int.). The peptide sequences are shown in the following Figs. 5 and 6. The amino acid sequence of Lys C fragment L20 (Fig. 5) demonstrates that the form of VEGF II A subunit in the heterodimer is the 164 amino acid form. The amino acid sequence of Lys C fragment L26 (Fig. 6) demonstrated that the form of VEGF II B subunit in the heterodimer is the 135 amino acid form.

EXAMPLE 9

Cloning and Sequencing of the VEGF II A Monomer

PCR Amplification, Cloning and Sequencing of R4238

Two degenerate oligonucleotides were synthesized in order to amplify the cDNA encoding the peptide sequences of VEGF A subunit between LysC fragment L42 and tryptic fragment T38. These oligonucleotides were:

L42: 2' 5' GTGAGACCTTCATGGAGTCGCTGTTATTCGCA 3' T388'B
5' CAGAGAGAACTGTCGACAAGTCGCTGTTATTCGCA 3'

where N=ACGT

Poly A⁺ RNA was isolated from GS-9L cells using the Fast Track RNA isolation kit from Invitrogen and the protocol provided. First strand cDNA synthesis was performed as follows:

1 μ l of GS-9L RNA was annealed to 1 μ l of adapter primer TAAT, 5' GACTGGAGTGGACATCGA-3' by incubating in a volume of 10 μ l at 70°C for 5 min, followed by cooling to room temperature. To this reaction was added:

3 μ l water
0.5 μ l 10X buffer (500 mM Tris-HCl pH 8.0, 750 mM KCl, 100 mM MgCl₂, 5 mM dithiothreitol)
0.5 μ l 100 mM DTT
0.5 μ l 10 mM each dATP, dGTP, dCTP, dTTP
0.5 μ l 15 units RNasin
0.5 μ l 40 mM Na pyrophosphate
1.5 μ l 10 U reverse transcriptase

and the reaction was incubated at 42°C for 1 hour, then diluted to 1 ml in 10 mM Tris-HCl, 1 mM EDTA, pH 7.5.

PCR Reactions

Reaction: 100 μ l

10 μ l 10X buffer from Perkin Elmer Cetus GeneAmp kit
10 μ l 2.5 mM each dATP, dGTP, dCTP, and dTTP
0.5 μ l forward and reverse primers
0.5 μ l 25 mM MgCl₂

2.1 50 pMoles T333'B
 0.5 ul 2.5 units Amplitaq DNA polymerase
 67.5 ul water
 Reaction conditions: 40 cycles of 94°C, 1'; 55°C, 2'; 72°C, 2'

Prep scale secondary reaction:

100 ul 10X buffer
 160 ul 1.25 mM each stock of dATP, dGTP, dGTP, and dTTP
 10 ul primary PCR reaction
 20 ul 500 pMoles L40.2
 20 ul 500 pMoles T333'B
 5 ul 2.5 units Amplitaq DNA polymerase
 665 ul water

Reaction conditions 94°C, 1'; 55°C, 2'; 72°C, 2'; 30 cycles.

The PCR product was concentrated by Centricon 30 spin columns, purified on a 1% agarose gel, and digested with restriction endonuclease Sall. The Sall fragment was then ligated into Sall cut pGEM3Z(+). The ligation mix was used to transform E. coli XL-1 blue. Plasmid DNA was isolated from white transformants and sequenced by the dideoxy chain termination method.

PCR Amplification, Cloning and Sequencing of pW-3

Based on the sequence obtained from the p4238 clones, two specific PCR primers were synthesized: oligo 307 5'-TTTGTGGACTCAGAGCGGAGAAAGC 3' and oligo 289 5'-TTTGTGGACGAAAATCACTGTGAGC 3'. These primers were used in combination with oligo A 17 5'-GACTCGAGTGGACATCG 3' to amplify the cDNA encoding the C-terminus of VEGF A subunit using the 3' RACE technique described by Frohman et al., PNAS 86: 8998-9002 (1989).

PCR reactions:

Primary reaction 100 ul

10 ul 10X buffer from Perkin Elmer Cetus
 GeneAmp kit
 18 ul 1.25 mM each stock of dATP, dGTP, dGTP, and dTTP
 0.35 ul first strand GS-9L cDNA
 1 ul 50 pMoles oligo 289
 0.5 ul 2.5 units Amplitaq DNA polymerase
 67.15 ul water

Reaction conditions 94°C, 1'; 58°C, 2'; 72°C, 2'; 10 cycles then add 50 pMoles A17, then 1 cycle of 94°C, 1'; 58°C, 2'; 72°C, 40' followed by 40 cycles 94°C, 1'; 58°C, 2'; 72°C, 2'.

Prep Scale secondary reaction:

60 ul 10X buffer
 108 ul 1.25 mM each stock of dATP, dGTP, dGTP, and dTTP
 24 ul primary PCR reaction
 12 ul 300 pMoles oligo 307
 12 ul 300 pMoles oligo A17
 5 ul 2.5 units Amplitaq DNA polymerase
 281 ul water

Reaction conditions 94°C, 1'; 58°C, 2'; 72°C, 2'; 30 cycles.

The PCR product was purified on a 1% agarose gel and digested with restriction endonuclease Sall. The Sall fragment was then ligated into Sall cut pGEM3Z(+). The ligation mix was used to transform E. coli XL-1 blue. Plasmid DNA was isolated from white transformants and sequenced by the dideoxy chain termination method.

PCR Amplification, Cloning and Sequencing of p8-18

Based on the sequence of p4238 clones, two specific PCR primers were synthesized: oligo 113 5'-TTTGTGGACAAAGTCAAGAGCGGCTTGAAGC 3' and oligo 74 5'-TTTGTGGACATACTGCTGGAAGATGTGGC 3'. These primers were used in combination with oligo A17 5'-GACTCGAGTGGACATCG 3' to amplify the cDNA encoding the C-terminus of VEGF A subunit using the 3' RACE technique described by Frohman et al., PNAS 86: 8998-9002 (1989). The PCR product was then ligated into Sall cut pGEM3Z(+). The ligation mix was used to transform E. coli XL-1 blue. Plasmid DNA was isolated from white transformants and sequenced by the dideoxy chain termination method.

RNA was isolated from GS-9L cells using the Fast Track RNA isolation kit from Invitrogen using the protocol provided. First strand cDNA synthesis was performed as follows:

One μg of GS9L RNA was annealed to 1 μg of oligo 151 by incubating in a volume of 6 μl at 70°C for 5' followed by cooling to room temperature. To this reaction was added:

- 5 1.5 μl 10X buffer (500mM Tris-HCl, pH 8.3,
750 mM KCl, 100 mM MgCl_2 , 5 mM spermidine)
- 2.5 μl 10 mM DTT
- 2.5 μl 10 mM each dATP, dGTP, dCTP, dTTP
- 0.6 μl 25 units RNasin
- 10 2.5 μl 40 mM Na pyrophosphate
- 9.5 μl 20 units diluted reverse transcriptase

The reaction was incubated at 42°C for 1 hour. Excess oligo 151 was removed by Centricon 100 spin columns and the 5' end of the cDNA was tailed by the addition of dATP and terminal transferase. The tailed cDNA was diluted to a final volume of 150 μl in 10 mM Tris-HCl, 1 mM EDTA, pH 7.5.

15 PCR Reactions:

Primary reaction (50 μl)

- 5 μl 10X buffer from Perkin Elmer Cetus GeneAmp Kit
- 8 μl 1.25 mM each stock of dATP, dCTP, dGTP, and dTTP
- 5 μl first strand GS-9L cDNA prime with oligo 151 and tailed
- 20 1 μl 25 pMoles oligo 113
- 1 μl 25 pMoles oligo A17
- 1 μl 10 pMoles oligo TA17
- 0.25 μl 1.25 units AmpliTaq DNA polymerase
- 23.75 μl water

25 Reaction conditions: 1 cycle 94°C 1'; 50°C 2'; 72°C 40' then 40 cycles of 94°C 1'; 50°C 1'30"; 72°C 2'

Prep scale secondary reaction:

- 50 μl 10X buffer
- 95 μl 1.25 mM each stock of dATP, dCTP, dGTP, and dTTP
- 6 μl primary PCR reaction
- 30 12 μl 300 pMoles oligo 74
- 12 μl 300 pMoles oligo A17
- 3 μl 15 units AmpliTaq DNA polymerase
- 411 μl water

 Reaction conditions 94°C, 1'; 55°C, 2'; 72°C, 2'30 cycles.

35 The PCR product was concentrated by Centricon 100 spin columns, and digested with restriction endonuclease Sall. The Sall fragment was then ligated into Sall cut pGEM3Z(+). The ligation mix was used to transform E. coli XL-1 blue. Plasmid DNA was isolated from white transformants and sequenced by the dideoxy chain termination method. The base sequence is shown in Fig. 5.

40 Cloning and sequencing of alternative forms of VEGF A cDNA

 Based on the sequence obtained from the p5-15 and pW-3 clones, two specific PCR primers were synthesized; oligo 5' C 5' TTTGTGACAAACCATGAACCTTCTGC 3' and oligo 181 5' TTTGTGACGGTGA-GAGGTCTAGTTTC 3'. These primers were used together to amplify multiple cDNAs encoding alternative forms of the VEGF A subunit.

45 Preparative PCR Reaction:

- 50 μl 10X buffer
- 80 μl 1.25mM each stock of dATP, dCTP, dGTP, and dTTP
- 10 μl first strand GS-9L cDNA
- 50 10 μl 300pMoles oligo 5' C
- 10 μl 300pMoles oligo 181
- 2.5 μl 15 units AmpliTaq DNA polymerase
- 337.5 μl water

 Reaction conditions 94°C, 1'; 58°C, 2'; 72°C, 3'; 40 cycles.

55 The PCR product was extracted with phenol/chloroform, concentrated by Centricon 30 spin columns, precipitated by ethanol, and digested with restriction endonuclease Sall I, and ligated into Sall cut pGEM3Z(+). The ligation mix was used to transform E. coli XL-1 blue. Plasmid DNA was isolated from white transformants and sequenced by the dideoxy chain termination method. Three sets of clones were identified. Clone #12

encoded the 164 amino acid secreted form of VEGF A subunit identical to that shown in Fig. 4. The 164 amino acid form of VEGF A subunit is that amino acid sequence running continuously from Ala³⁷ to Arg¹⁹⁰. Clone #14 has a 135 base pair deletion between the second base of the Asn¹⁴⁹ codon and the third base of the Arg¹⁹⁰ codon. This clone thus encodes a 120aa secreted form of the VEGF A subunit with the conversion of Asn¹⁴⁹ to Lys¹⁴⁹. The 120 amino acid form of VEGF A subunit runs from Ala³⁷ to Asn¹³⁹, which becomes Lys¹⁴⁹ and does not begin until Cys¹³⁵; this form also finishes at Arg¹⁹⁰. Clone #16 has a 72 base pair insertion between the second and third base of the Asn¹⁴⁹ codon. This clone thus encodes a 138 amino acid secreted form of the VEGF A subunit with the conversion of Asn¹⁴⁹ to Lys¹⁴⁹. The nucleotide sequence and the deduced amino acid sequence of this insertion is:

Lys Lys Ser Val Arg Gly Lys Gly Lys Gly Gln Lys
A AAA TCA GTT CGA GGA AAG GGA AAG GGT CAA AAA

Arg Lys Arg Lys Lys Ser Arg Phe Lys Ser Trp Ser
CGA AAG CGC AAG AAA TCC CGG TTT AAA TCC TGG AGC

Val

GT

EXAMPLE 10

Cloning and Sequencing of the VEGF II B Subunit

PCR Amplification, Cloning and Sequencing of pYG

Two degenerate oligonucleotides were synthesized in order to amplify the cDNA encoding the peptide sequences of VEGF II B on Lys C fragment L50. These oligonucleotides were:

Y1 5' TTTGTGACATA[TC]AT[TC]GC[N]GA[TC]GA[AG]C 3'
GC 5' TTTGTGACTC[AG]TC[AG]TT[AG]CA[AG]CA[N]CC 3'

where N=ACGT

RNA was isolated from GS-9L cells using the Fast Track RNA isolation kit from Invitrogen and the protocol provided. First strand cDNA synthesis was performed as follows:

1 µg of GS-9L poly A⁺RNA was annealed to 1 µg of adapter primer TA17, 5'-GACTGAGTGGACATC-GATTTT-3', by incubating in a volume of 10 µl at 70°C for 5 min, followed by cooling to room temperature. To this reaction was added:

3.0 µl water
2.5 µl 10X buffer (500 mM Tris-HCl, pH 8.3, 750 mM KCl, 100 mM MgCl₂, 5 mM spermidine)
2.5 µl 100 mM DTT
2.5 µl 10 mM each dATP, dGTP, dCTP, dTTP
0.8 µl 15 units RNasin
2.5 µl 40 mM Na pyrophosphate
1.5 µl 15 units reverse transcriptase

and the reaction was incubated at 42°C for 1 hour, then added to 1 ml of 10 mM Tris-HCl, 1 mM EDTA, pH 7.5.

PCR Reactions

Primary reaction (50 µl):

5 µl 10X buffer from Perkin Elmer Cetus GeneAmp kit
8 µl 1.25 mM each stock of dATP, dGTP, dCTP, and dTTP
1 µl first strand GS-9L cDNA
1 µl 50 pMoles of each primer
1 µl 50 pMoles of goTaq
0.25 µl 1.25 Unit Amp Taq DNA polymerase

33.75 μ l water

Reaction conditions, 40 cycles of 94°C, 1'; 50°C, 2'; 72°C, 2'.

Prep scale reaction

60 μ l 10X buffer

96 μ l 1.25mM each stock of dATP, dCTP, dGTP, and dTTP

12 μ l first strand 659L cDNA

12 μ l 500pMoles oligo Y1

12 μ l 500pMoles oligo GC

3 μ l 15 units Amplitaq DNA polymerase

405 μ l water

Reaction conditions 94°C, 1'; 50°C, 2'; 72°C, 2' 40 cycles.

The PCR product was concentrated by Centricon 30 spin columns and digested with restriction endonuclease Sall. The Sall fragment was then ligated into Sall cut pGEM3Zf(+). The ligation mix was used to transform *E. coli* XL-1 blue. Plasmid DNA was isolated from white transformants and sequenced by the dideoxy chain termination method.

PCR Amplification, Cloning and Sequencing of p3V2

Based on the sequence obtained from the pYG clones, a specific PCR primer was synthesized; oligo HP 5' TTTGTGCGACACACCCTAATGAAGTGC 3'. This primer was used in combination with oligo A17 5' GACTCGAGTCGACATCG 3' to amplify the cDNA encoding the COOH terminus of the VEGF II B subunit using the 3' RACE technique described by Frohman *et al.*, PNAS 85: 8998-9002 (1988).

Preparative PCR reaction:

60 μ l 10X buffer from Perkin Elmer Cetus Gene Amp Kit

12 μ l first strand 659L cDNA

96 μ l 1.25 mM each of dATP, dCTP, dGTP, dTTP

12 μ l 300 pMoles oligo A17

12 μ l 300 pMoles oligo HP

3 μ l 15units Amplitaq DNA polymerase

405 μ l water

Reaction conditions 1 cycle of 94°C, 1'; 58°C, 2'; 72°C, 2'; followed by 40 cycles 94°C, 1', 58°C, 2' and 72°C, 2'.

The PCR product was concentrated by Centricon 30 spin columns, precipitated with ethanol and digested with restriction endonuclease Sall. The Sall fragment was then ligated into Sall cut pGEM3Zf(+). The ligation mix was used to transform *E. coli* XL-1 blue. Plasmid DNA was isolated from white transformants and sequenced by the dideoxy chain termination method.

PCR Amplification, Cloning and Sequencing of p5V2

Based on the sequence of pYG clones, two specific PCR primers were synthesized; oligoVL' 5' TTTGTGCGACACAGCGACTCAGAAGG 3' and oligoVS' 5' TTTGTGCGACACTGAATATATGAGACAC 3'. These primers were used in combination with oligo A17 5' GACTCGAGTCGACATCG 3' to amplify the cDNA encoding the amino terminus of the VEGF II B subunit using the 5' RACE technique described by Frohman *et al.*, supra. Oligo 151' was synthesized in order to prime cDNA from GS-9L RNA. Oligo 151' is 5' CTTCATCATTGCAGCAGC 3'.

Poly A⁺RNA was isolated from GS9L cells using the Fast Track RNA isolation kit from Invitrogen using the protocol provided. First strand cDNA synthesis was performed as follows:

One μ g of GS9L RNA was annealed to 1 μ g of oligo 151' by incubating in a volume of 5 μ l at 70°C for 5' followed by cooling to room temperature. To this reaction was added:

1.5 μ l 10X buffer (500 mM Tris-HCl, pH 8.3,

750 mM KCl, 100 mM MgCl₂, 5mM spermidine)

2.5 μ l 10 mM DTT

2.5 μ l 10 mM each dATP, dGTP, dCTP, dTTP

0.5 μ l 25 units RNasin

2.5 μ l 40 mM Na pyrophosphate

9.5 μ l 20 units diluted reverse transcriptase

The reaction was incubated at 42°C for 1 hour.

Excess oligo 151' was removed by Centricon 100 spin columns and the 5' end of the cDNA was tailed by

the addition of dATP and terminal transferase. The tailed cDNA was diluted to a final volume of 150 μ l in 10 mM Tris-HCl, 1 mM EDTA, pH 7.5

PCR Reactions:

Primary reaction (50 μ l)

- 5 μ l 10X buffer from Perkin Elmer Cetus GeneAmp Kit
- 8 μ l 1.25 mM each stock of dATP, dCTP, dGTP, and dTTP
- 5 μ l first strand GS9L cDNA primed with oligo 151 and tailed
- 1 μ l 25 pMoles oligo VL'
- 1 μ l 25 pMoles oligo A17
- 10 1 μ l 10 pMoles oligo TA17
- 0.25 μ l 1.25 units AmpliTaq DNA polymerase
- 28.75 μ l water
- Reaction conditions: 1 cycle 94°C, 1'; 58°C, 2'; 72°C, 40' then 40 cycles of 94°C, 1'; 58°C, 2'; 72°C, 2'.

Prep scale secondary reaction:

- 15 100 μ l 10X buffer
- 160 μ l 1.25 mM each stock of dATP, dCTP, dGTP, and dTTP
- 10 μ l primary PCR reaction
- 20 μ l 500 pMoles oligo VS'
- 20 μ l 300 pMoles oligo A17
- 20 5 μ l 25 units AmpliTaq DNA polymerase
- 685 μ l water
- Reaction conditions 94°C, 1'; 58°C, 2'; 72°C, 2'30 cycles.

The PCR product was extracted with phenol/chloroform, concentrated by Centricon 30 spin columns, pre-precipitated by ethanol, and digested with restriction endonuclease SalI. The SalI fragment was purified on 4% Nu-Sieve Agarose gel then ligated into SalI cut pGEM3Zf(+). The ligation mix was used to transform *E. coli* XL-1 blue. Plasmid DNA was isolated from white transformants and sequenced by the dideoxy chain termination method.

PCR Amplification, Cloning and Sequencing of pCV2 and pCV2.1

30 Based on the sequences of the p3V2 and p5CV2 clones, two specific PCR primers were synthesized; oligo 5'CV2, 1 5' TTTGTGCGAC[N][N]GCAGGTCTAGCTG 3' and oligo 3'CV2 5' TTTGTGCGAC[N][N]CTAATAAA-TAGAGGG 3'. These primers were used together to amplify the cDNA encoding the VEGF B subunit.

Preparative PCR Reaction:

- 35 40 μ l 10X buffer
- 64 μ l 1.25 mM each d.ATP, dTTP, dGTP, dCTP
- 8 μ l first strand GS-9L cDNA
- 8 μ l 200 pMoles 5'CV2.1
- 8 μ l 200 pMoles 3'CV2
- 40 2 μ l 10units AmpliTaq DNA polymerase
- 270 μ l water
- Reaction conditions: 94°C, 1'; 58°C, 2'; 72°C, 2'; 40 cycles.

The PCR product was extracted with phenol/chloroform, concentrated by Centricon 30 spin columns, pre-precipitated by ethanol, and digested with restriction endonuclease SalI, and ligated into SalI cut pGEM3Zf(+). 45 The ligation mix was used to transform *E. coli* XL-1 blue. Plasmid DNA was isolated from white transformants and sequenced by the dideoxy chain termination method. Two sets of clones were identified, one encoded a 135 amino acid sequence and the other encoded a 115 amino acid sequence, see Figures 6 and 7 respectively.

cDNA Cloning of VEGF B Subunit

50 The DNA and protein sequences for the amino terminus of the signal peptide of VEGF B was determined from a cDNA clone isolated from a cDNA library constructed from GS-9L polyA⁺ RNA.

First Strand Synthesis

- 55 Anneal 15.6 μ l (5ug) GS-9L polyA⁺ RNA and 2.5 μ l (2.5ug) oligo dT-XbaI primer by heating to 70° C 5' slow cool to room temperature. Add the following:
- 5.5 μ l 10X buffer (500 mM Tris-HCl, pH 8.3 (42° C),
- 750 mM KCl, 100 mM MgCl₂, 5mM spermidine
- 5.5 μ l 100mM DTT

5.5 μ l 10 mM each dATP, dTTP, dCTP, dGTP

1.4 μ l (55units) RNasin

5.5 μ l 40mM NaPPi

13.5 μ l 55units AMV reverse transcriptase

Incubate at 42° C 60'.

Second Strand Synthesis:

Assemble reaction mix

50 μ l first strand reaction

25 μ l 10X buffer (500 mM Tris-HCl, pH7.2, 850 mM KCl, 30 mM MgCl₂ 1mg/ml BSA, 100 mM (NH₄)₂SO₄)

7.5 μ l 100 mM DTT

25 μ l 1mM NAD

6.5 μ l (65units) *E. coli* DNA Polymerase I

2.5 μ l (2.5units) *E. coli* DNA Ligase

2.5 μ l (2 units) *E. coli* RNase H

135 μ l water

Incubate at 14° C for 2h and then incubate 70° C for 10'. Add 1 μ l (10 units) T4 DNA Polymerase, incubate at 37° C for 10', add 25 μ l 0.2M EDTA an extract with phenol/chloroform, then precipitate by the addition of 0.5 volume of 7.5 M ammonium acetate and 3 volumes of ethanol, collect precipitate and resuspend in 20 μ l of 10 mM Tris-HCl, pH 7.5, 1mM EDTA.

cDNA Library Construction

The above cDNA was ligated into EcoR1/ XbaI digested LambdaGEM-4 (Promega Biochemicals) after the addition of EcoR1 linkers and digestion with EcoR1 and XbaI. A cDNA library was amplified from ~50, 000 independent clones.

Isolation of Rat VEGF B cDNA Clone

The above cDNA library was screen by plaque hybridization using pCV2 as a probe. Hybridization conditions were as follows:

5XSSC (1XSSC is 0.15M sodium chloride, 0.015M sodium citrate,

50% Formamide

5X Denhardt's Solution (1% Ficoll, 1% polyvinylpyrrolidone, 1% bovine serum albumin)

0.15 mg/ml salmon sperm DNA hybridize overnight at 42° C.

Filters were washed 3 times in 2XSSC, 0.1% SDS at room temperature for 5', then 1 time in 1XSSC, 0.1% SDS at 50C for 30'. Positive clones were identified by autoradiography.

The DNA from phage #202 was digested with restriction endonuclease SpeI and the 1.1kb band ligated into XbaI digested pGEM3Zf(+). The ligation mix was used to transform *E. coli* XL-1 blue. Plasmid DNA was isolated from white transformants and sequenced by the dideoxy chain termination method. The cDNA sequence and predicted amino acid sequence of the signal peptide are shown in Fig.6 and Fig.7.

The entire nucleotide and amino acid sequence of the 115 amino acid form is shown in Fig. 7. The secreted protein starts at Ala²⁴ and continues to Arg¹²⁸. The entire nucleotide and amino acid sequence of the 135 amino acid form is shown in Fig. 7. The secreted protein starts at Ala²⁴ and continues to Leu¹³².

Claims

1. Mammalian vascular endothelial growth factor II comprising a heterodimer with an apparent non-reduced molecular mass of about 58,000 daltons, with said heterodimer made up of an A subunit having an apparent reduced molecular mass of about 23,000 daltons and a B subunit having an apparent reduced molecular mass of about 23,000 daltons with said vascular endothelial growth factor substantially free of impurities and mitogenic for mammalian vascular endothelial cells.
2. The vascular endothelial growth factor II of claim 1 wherein the growth factor is isolated and purified from human cells, cell culture media or tissues.
3. The vascular endothelial growth factor II of claim 1 wherein the factor is mitogenic for mammalian vascular endothelial cells.
4. Mammalian vascular endothelial growth factor II comprising a heterodimer with an apparent non-reduced molecular mass of about 58,000 daltons, with said homodimer made up of two A subunits having an apparent reduced molecular mass of about 23,000 daltons each with said vascular endothelial growth factor

substantially free of impurities and mitogenic for mammalian vascular endothelial cells.

- 5 5. Mammalian vascular endothelial growth factor comprising a homodimer comprising two B subunits with said endothelial growth factor substantially free of impurities and mitogenic for mammalian vascular endothelial cells.
- 10 6. A protein comprising an amino acid sequence substantially corresponding to all or a portion of the amino acid sequence of rat vascular endothelial growth factor II with said vascular endothelial growth factor having a B subunit selected from the group consisting of the 135 amino acid form and the 115 amino acid form and having an A subunit selected from the group consisting of the 188 amino acid form, the 164 amino acid form and the 120 amino acid form and other microheterogeneous forms thereof with said growth factor being free of other proteins.
- 15 7. Vascular endothelial growth factor comprising an A subunit of at least the amino acid sequence as shown in Figure 4 and a B subunit of at least the amino acid sequence as shown in Figure 5 and any microheterogeneous or truncated forms thereof which are biologically active and are substantially free of impurities.
- 20 8. Vascular endothelial growth factor comprising an A subunit of at least the nucleotide sequence as shown in Figure 4 and an B subunit of at least the nucleotide sequence as shown in Figure 6 and any microheterogeneous or truncated forms thereof which are biologically active and are substantially free of impurities.
- 25 9. Vascular endothelial growth factor comprising two B subunits of at least the nucleotide sequence as shown in figure 6 or figure 7 and any microheterogeneous or truncated forms thereof which are biologically active and are substantially free of impurities.
- 30 10. A process for the isolation of vascular endothelial growth factor II in substantially pure form which comprises the sequence of steps of:
 - a. isolation of conditioned growth media;
 - b. cation exchange chromatography;
 - c. lectin affinity chromatography;
 - d. cation exchange high performance liquid chromatography;
 - e. metal-chelate affinity chromatography;
 - 35 f. reverse-phase high performance liquid chromatography; and
 - g. collecting the substantially pure vascular endothelial growth factor II.
- 40 11. The process of claim 10 wherein:
 - a. isolation of conditioned growth media by filtration;
 - b. cation exchange chromatography with adsorption on carboxymethyl Sephadex C-50 cation exchange resin at pH 6.0 followed by a rinse with 0.05 M sodium phosphate, pH 6.0, 0.15 M NaCl and elution with 0.05 sodium phosphate, pH 6.0, containing 0.6 M NaCl;
 - c. lectin affinity chromatography with adsorption on concanavalin A agarose resin at pH 6.0 followed by elution with 0.05 M sodium acetate, pH 6.0, containing 1 mM CaCl_2 , 1 mM MnCl_2 , 0.1 M NaCl and 0.32 M alpha-methyl mannoside and 0.28 M alpha-methyl glucoside;
 - 45 d. cation exchange high performance liquid chromatography with adsorption on Polyaspartic Acid WDX, equilibrated in 0.05 M sodium phosphate, pH 6.0 followed by elution with about 0 to about 0.75 M NaCl linear gradient;
 - e. metal-chelate chromatography with adsorption on Pharmacia Chelating Sepharose 6B charged with excess Cu^{++} followed by elution with a gradient of 0.5 mM to 100 mM imidazole in buffer containing about 0.05 M sodium phosphate, about pH 7.0, about 2 M NaCl;
 - 50 f. reverse phase high performance liquid chromatography with adsorption on a C_{18} column equilibrated in 0.1 % trifluoroacetic acid followed by elution with a gradient of 0 to 57 % v/v of acetonitrile in 0.1 % trifluoroacetic acid;
 - 55 g. collecting the substantially pure vascular endothelial growth factor II.
12. The process of claim 10 for the isolation of vascular endothelial growth factor II from GS-61 cells in culture.

13. A tissue repairing pharmaceutical composition comprising a pharmaceutical carrier and an effective tissue repairing amount of the purified vascular endothelial growth factor II of any one of Claims 1 or 4 to 9.
14. The use of the vascular endothelial growth factor II of any one of Claims 1 or 9 to 9 for the manufacture of a medicament capable of promoting tissue repair.
15. A method for the stimulation of growth of vascular endothelial cells which comprises treating a sample of the desired endothelial cells in a nutrient medium with vascular endothelial growth factor II at a concentration of about 0.1-100 ng/ml.
16. A method for preparing synthetic vessels comprising treating synthetic polymeric vessels with vascular endothelial growth factor II.
17. The use of a synthetic vessel prepared by the method of Claim 16 for the manufacture of a medicament suitable for use as a vessel implant whereby, after implantation, endothelial cells migrate into and grow on the artificial surface.
18. The use of vascular endothelial growth factor II for the manufacture of a medicament for the stimulation of the growth of vascular endothelial cells in vivo whereby either vascular repair, or neovascularization, or both, is promoted.
19. A purified and isolated DNA sequence encoding the 188 amino acid A subunit of vascular endothelial growth factor II.
20. A purified and isolated DNA sequence encoding the 164 amino acid A subunit of vascular endothelial growth factor II.
21. A purified and isolated DNA sequence encoding the 120 amino acid A subunit of vascular endothelial growth factor II.
22. A purified and isolated DNA sequence encoding the 135 amino acid B subunit of vascular endothelial growth factor II.
23. A purified and isolated DNA sequence encoding the 115 amino acid B subunit of vascular endothelial growth factor II.
24. Vascular endothelial growth factor II DNA comprising an A subunit DNA sequence selected from the group consisting of a DNA sequence encoding an 188 amino acid form, a DNA sequence encoding an 164 amino acid form and a DNA sequence encoding a 120 amino acid form, with said A subunit DNA operably attached to a B subunit DNA sequence selected from the group consisting of a DNA sequence encoding a 135 amino acid form and a DNA sequence encoding a 115 amino acid form.
25. Heterodimeric vascular endothelial growth factor DNA comprising an A subunit DNA sequence encoding a 164 amino acid form operably attached to a B subunit DNA sequence encoding a 135 amino acid form.
26. Heterodimeric vascular endothelial growth factor DNA comprising an A subunit DNA sequence encoding a 164 amino acid form operably attached to a B subunit DNA sequence encoding a 115 amino acid form.
27. Homodimeric vascular endothelial growth factor DNA comprising a B subunit DNA sequence selected from the group consisting of a B subunit DNA sequence encoding a 135 amino acid form and a B subunit DNA sequence encoding a 115 amino acid form.
28. A vector containing the DNA sequence of any one of claims 24 to 27.
29. A host cell transformed by the vector of claim 28 containing the DNA sequence encoding vascular endothelial growth factor II.
30. A process for the preparation of vascular endothelial growth factor I homodimer and dimer using the transformed

host cell of claim 30 under conditions suitable for the expression of vascular endothelial growth factor II and recovering vascular endothelial growth factor II.

31. Vascular endothelial growth factor II made by the process of claim 30

5
10
15
20
25
30
35
40
45
50
55

FIG. 1

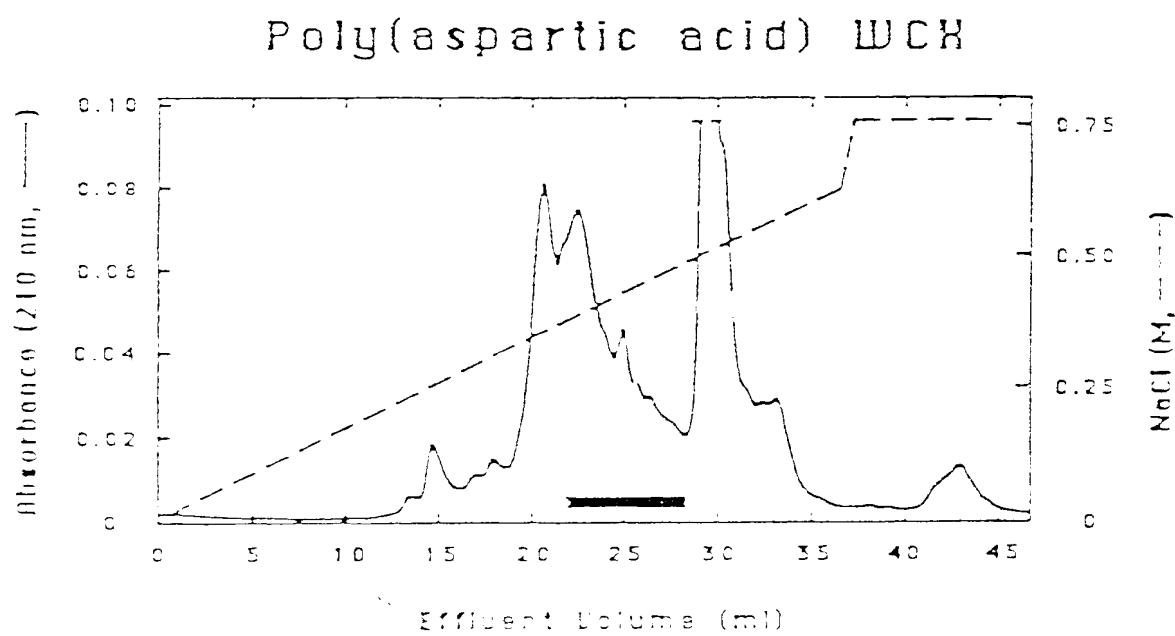


FIG. 2

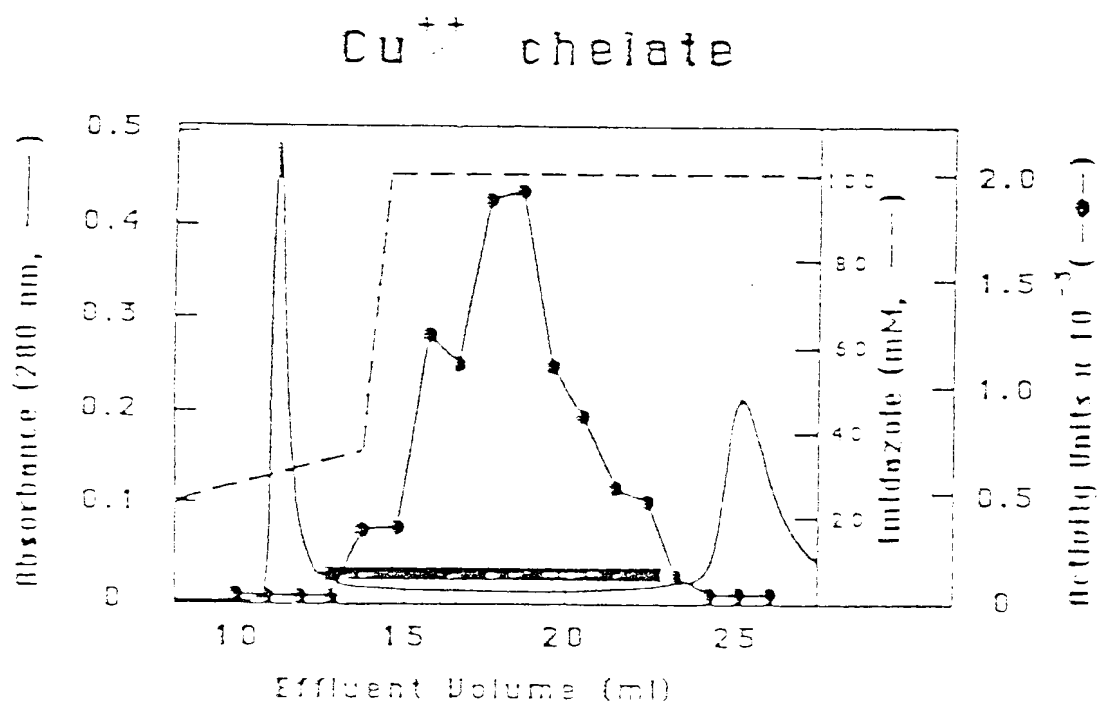


FIG. 3

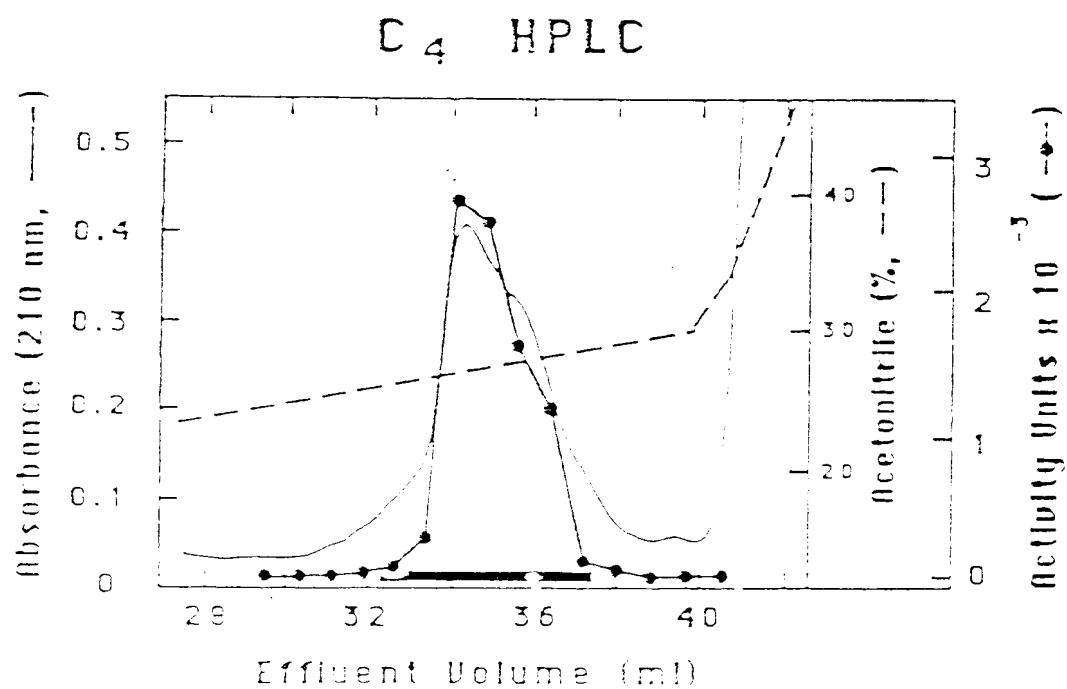


FIG. 4

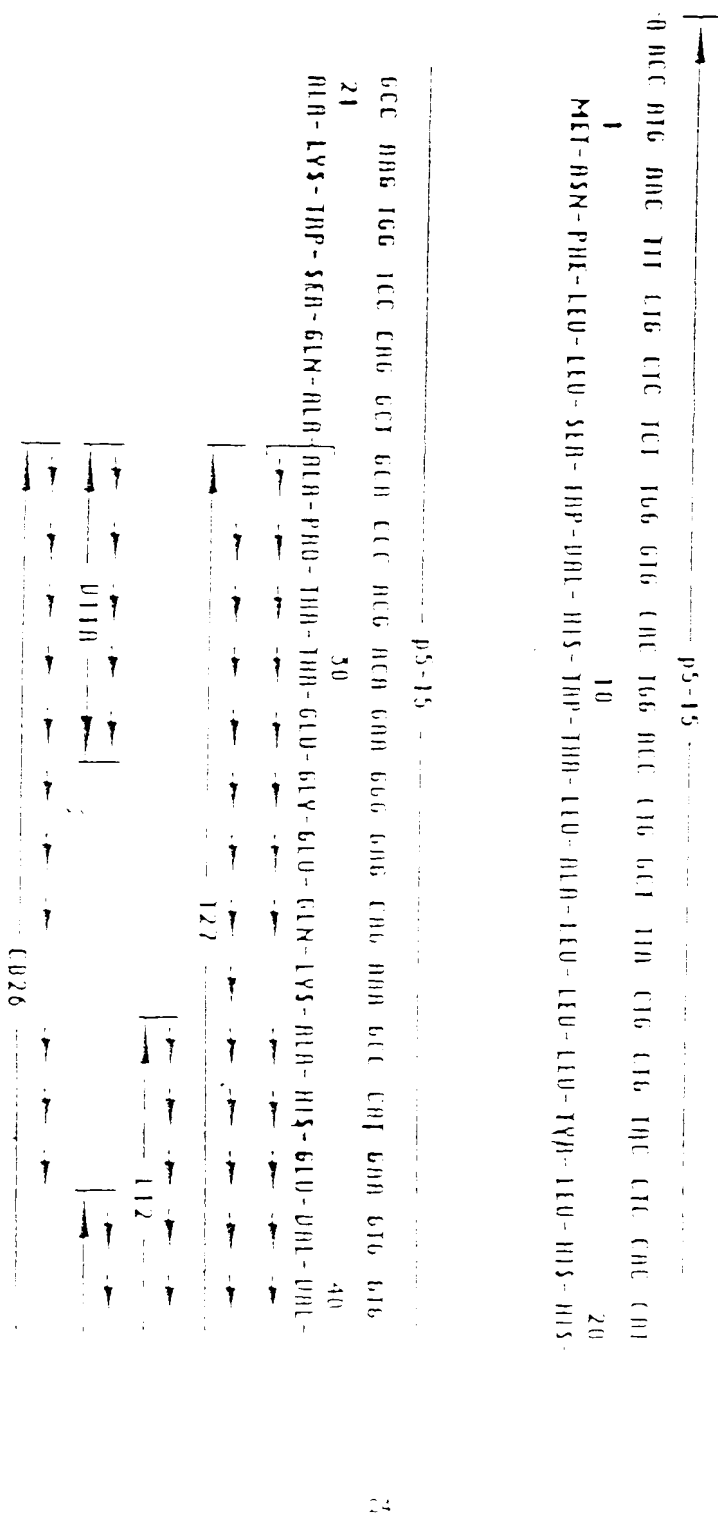


FIG. 4 (CONT.)

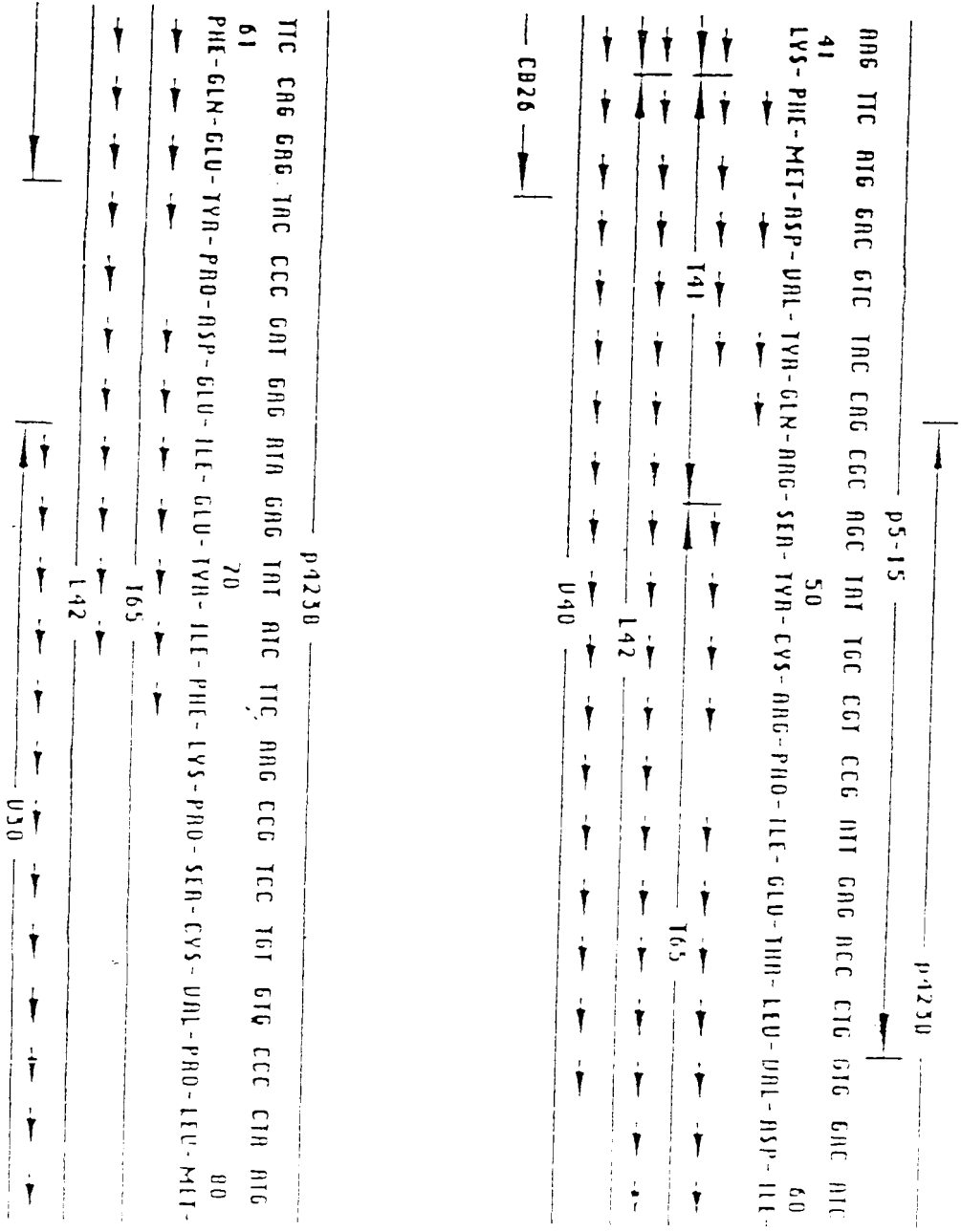


FIG. 4 (CONT.)

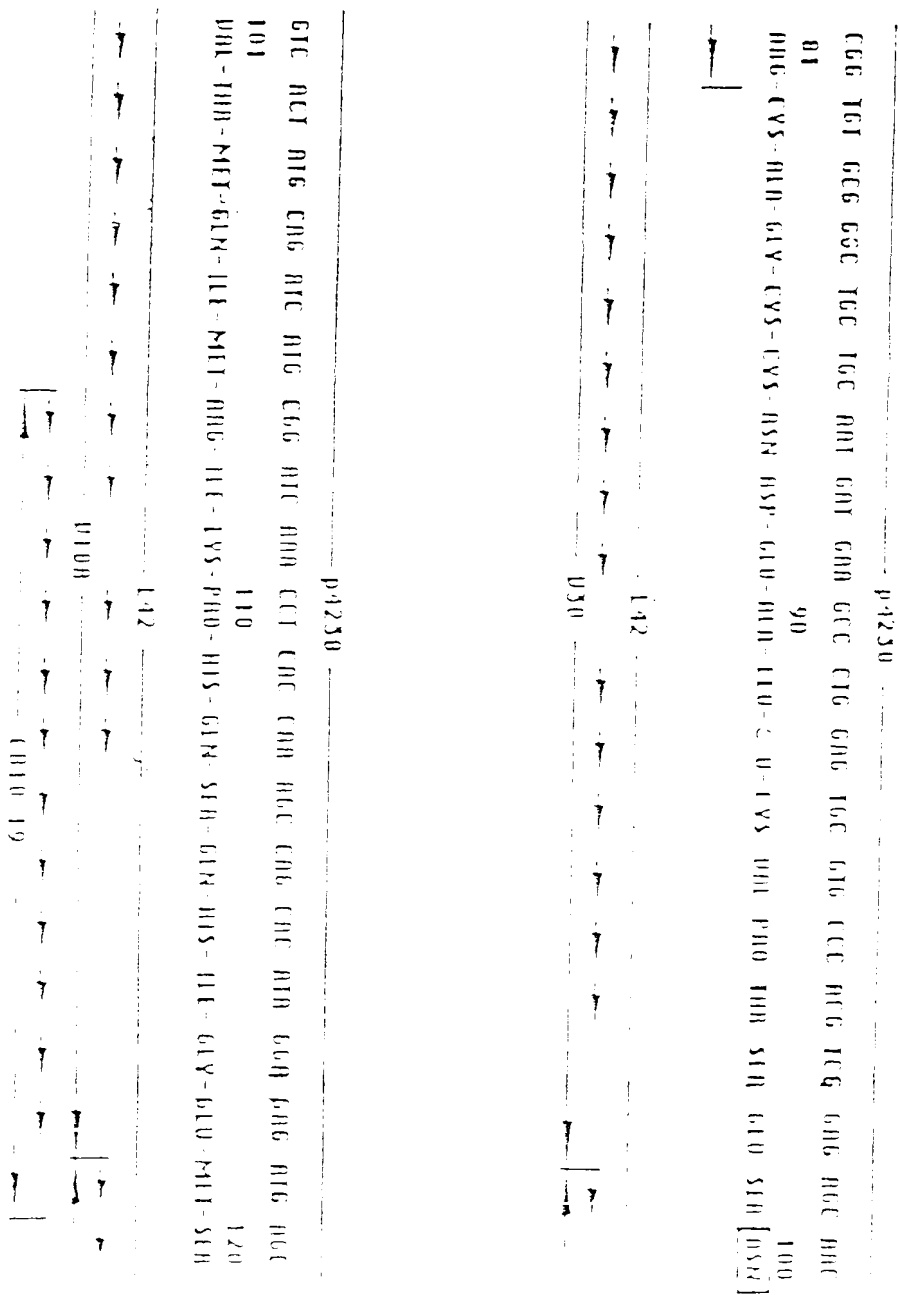
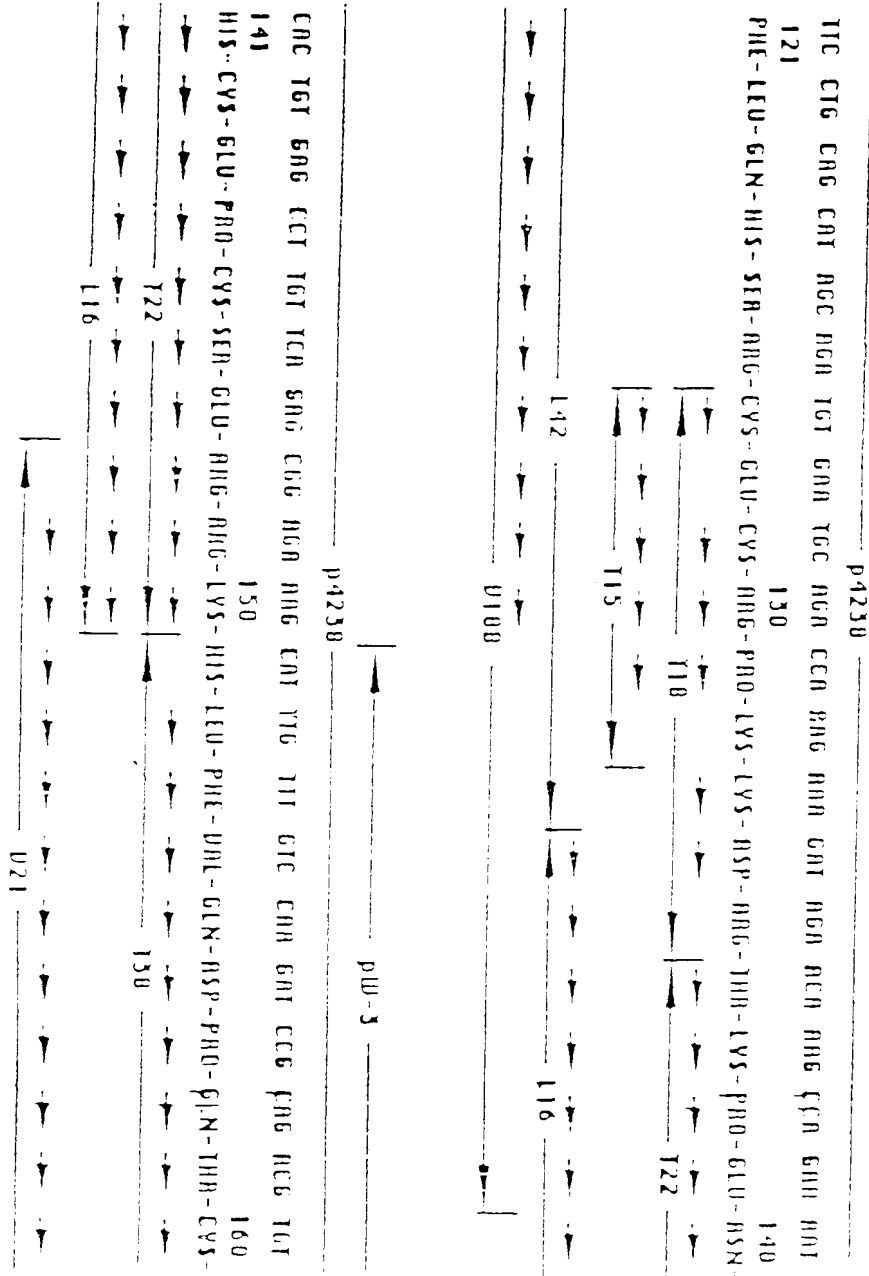


FIG. 4 (CONT.)



23

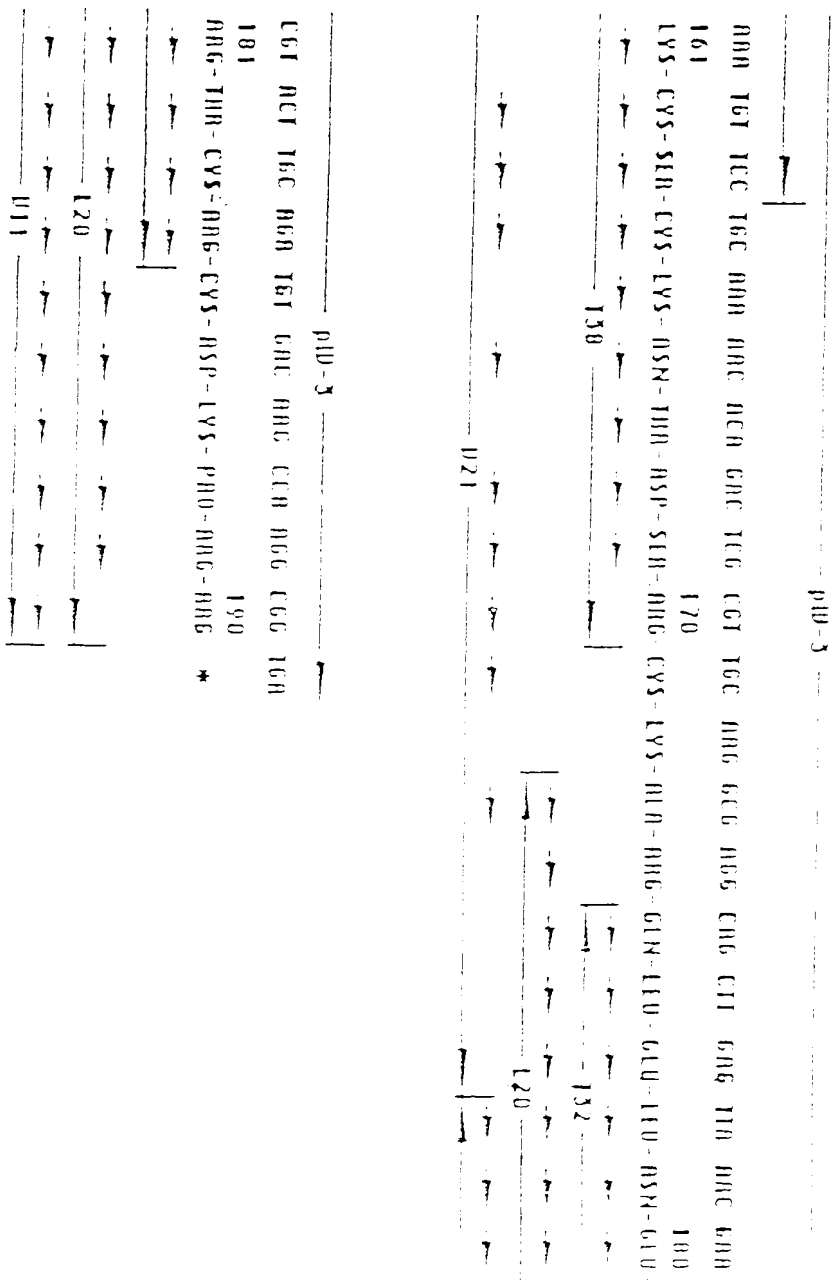


FIG. 5

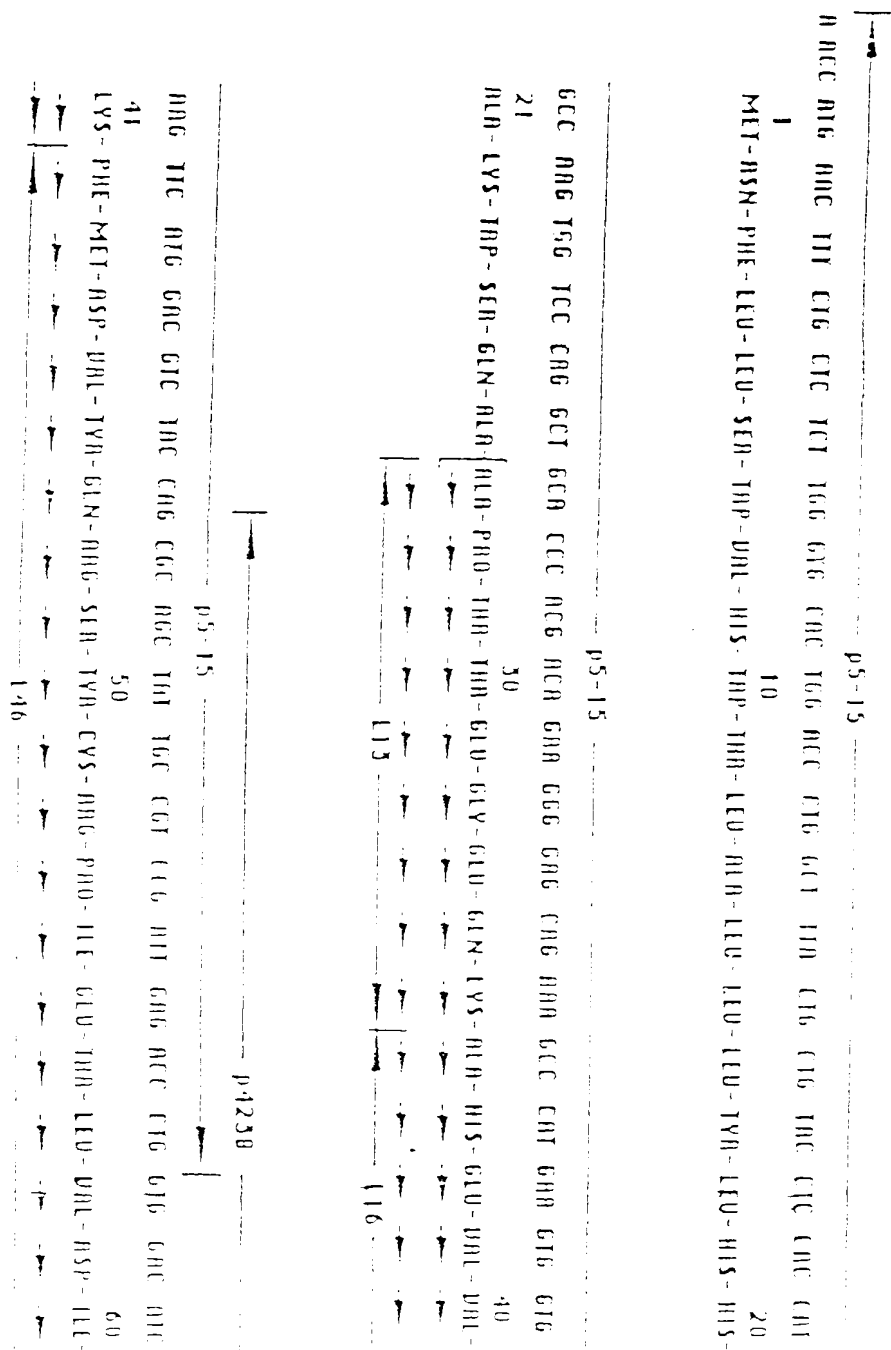


FIG. 5 (CONT.)

p4230
TTC CAG GAG TAC CCC GAT GAG ATG GAG TAT ATC TTC AAG CCG TCC TGT GTG CTC CTA ATG
61
PHE-GLN-GLU-TYR-PHE-ASP-GLU-ILE-GLU-TYR-ILE-PHE-LYS-PHE-SER-CYS-VAL-PHE-LEU-MET-
70
146

p4230
CGG TGT GCG GGC TGC TGC AAT GAT GAA GGC CTG GAG TGC GTG CCC ACC TCG GAG AGC AAC
81
AAG-CYS-GLN-GLY-CYS-CYS-ASN-ASP-GLU-ALA-LEU-GLU-CYS-VAL-PHE-SER-GLU-SER
90
100
146

p4230
GTC ACT ATG CAG ATC ATG CCG ATC AAA CCT CAC CAA AGC CAG CAC AAT GGA GAG ATG AGC
101
VAL-THR-MET-GLN-ILE-MET-AAG-ILE-LYS-PHE-HIS-GLN-SER-GLN-HIS-ILE-GLY-GLU-MET-SER-
110
120
146

FIG. 5 (CONT.)

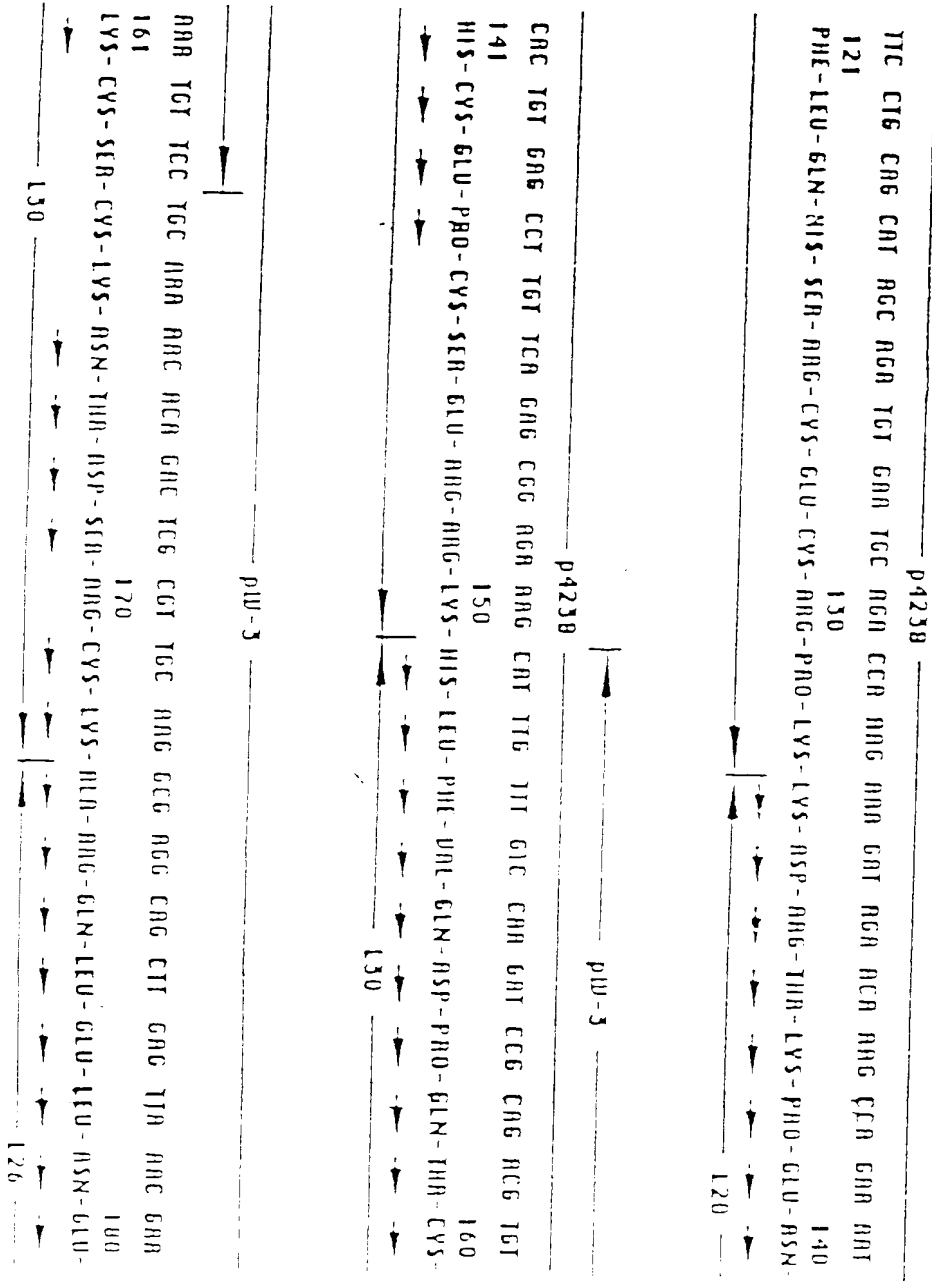


FIG. 5 (CONT.)

	pu-5	
CGT ACT TGC AGA TGT GAC AAG CCA AAG CCG TCA		
101		
AAG-TAA-CYS-AAG-CYS-ASP-LYS-PRO-AAG-AAG		
190		
+		

FIG. 6

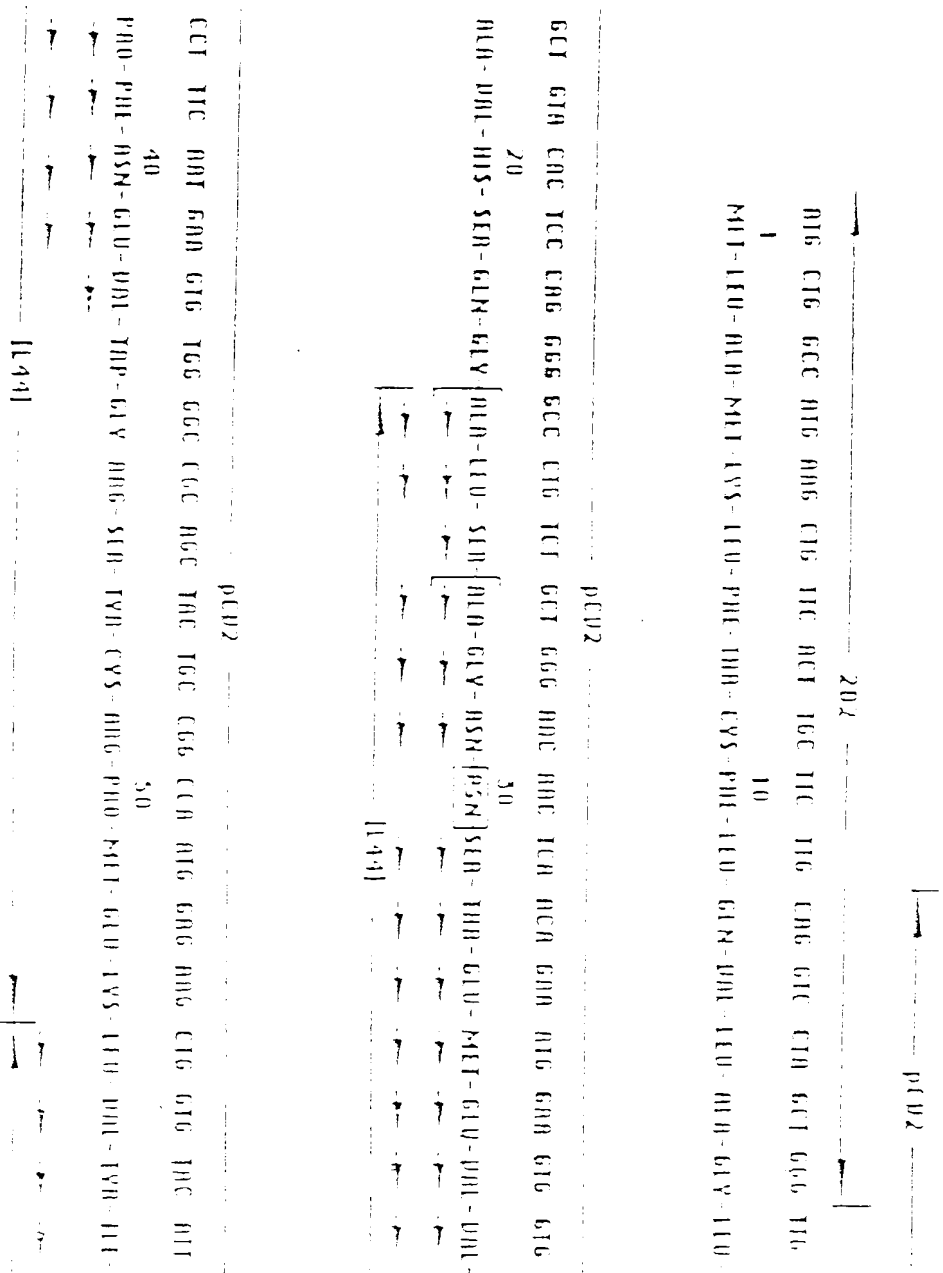


FIG. 6 (CONT.)

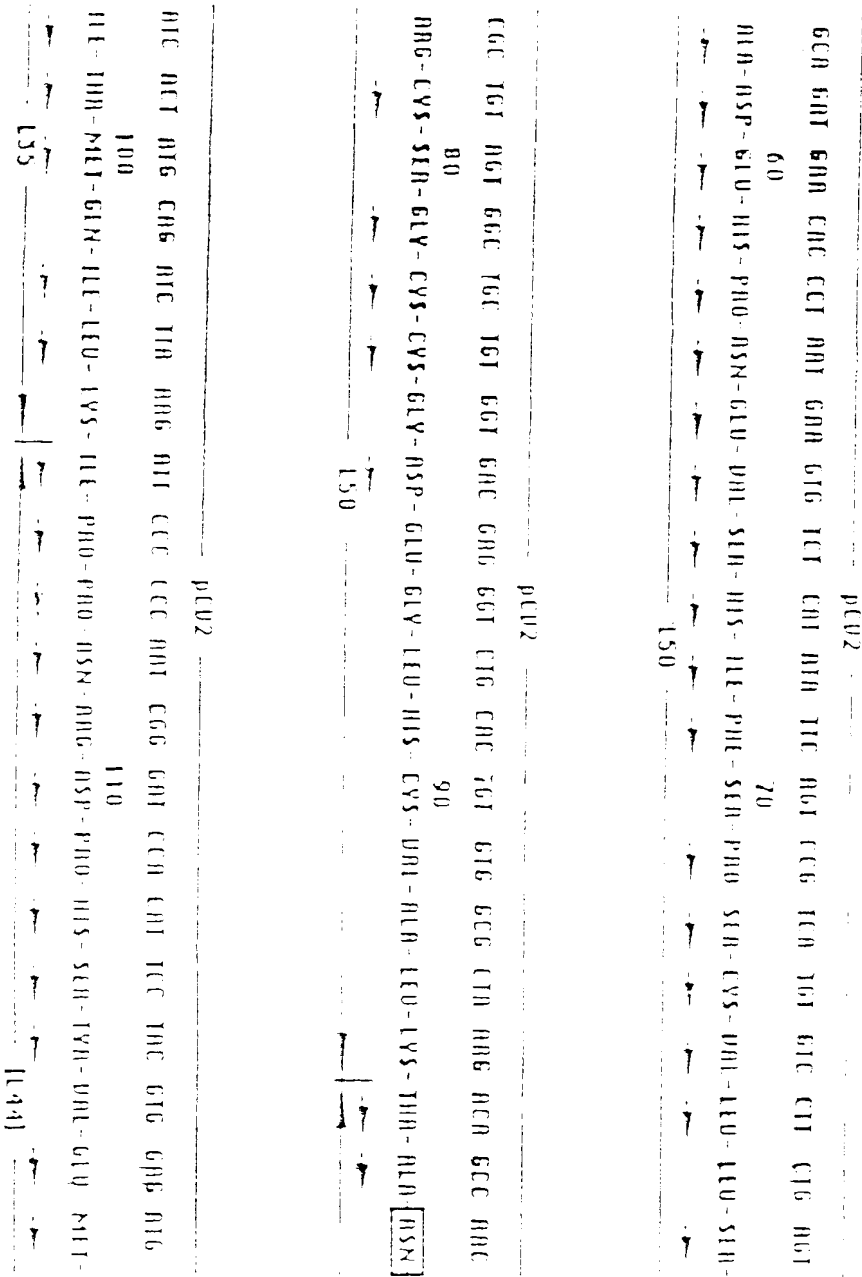
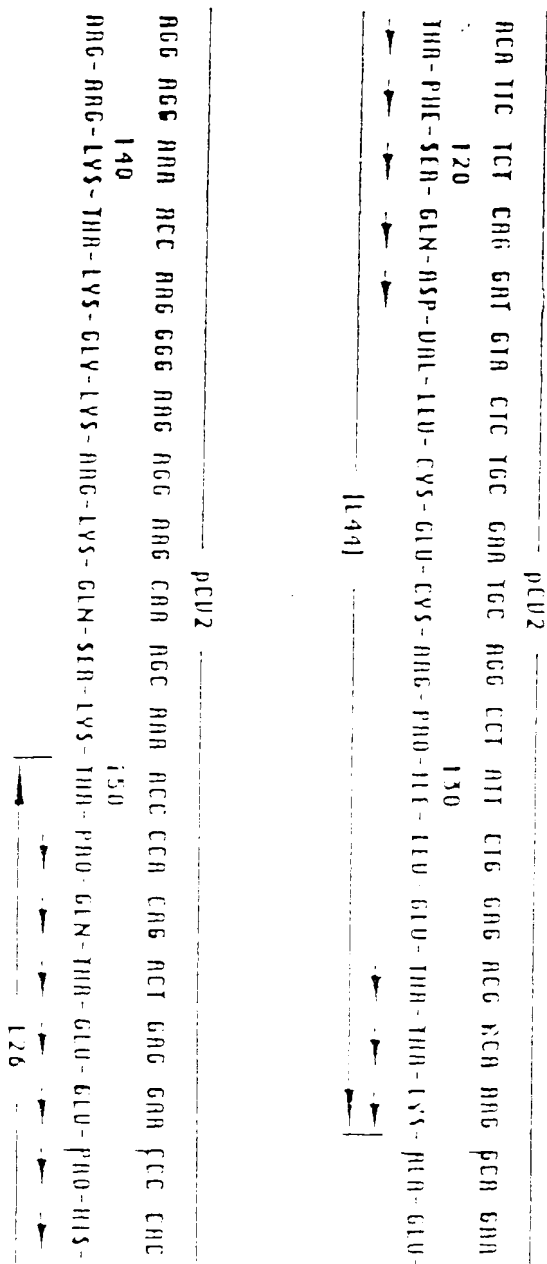


FIG. 6 (CONT.)



CTG TGA
 158
 LEU

FIG. 7

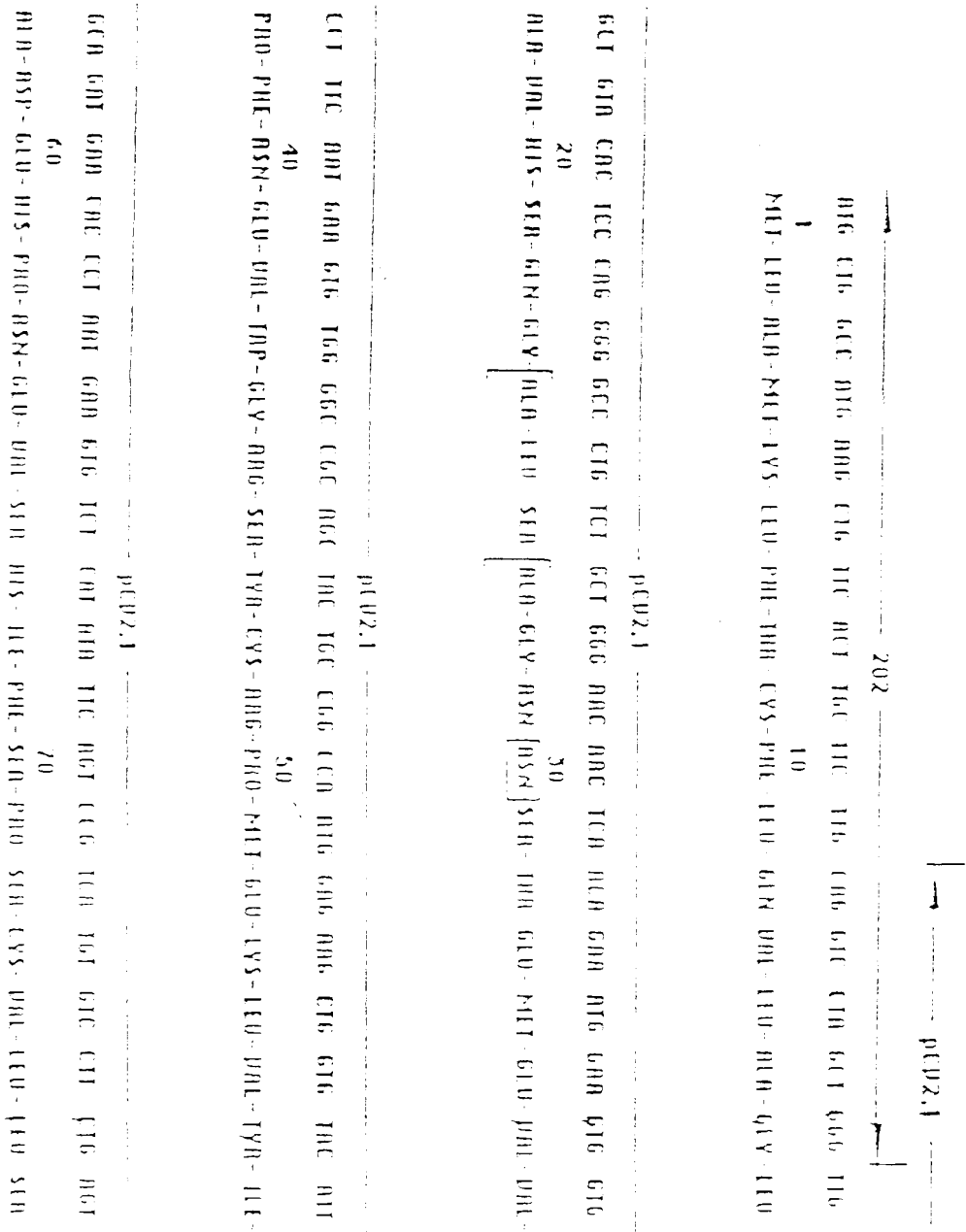


FIG. 7 (CONT.)

PCU2.1
 CGC TGT AGT GGC TGC TGT GGT GAC GAG GGT CTG CAC TGT GTG GCG CTA AAG ACC ACC ACC
 80
 AAG-CYS-SEN-GLY-CYS-CYS-GLY-ASP-GLU-GLY-LEU-HIS-CYS-CYS-DNL-ALA-LEU-LYS-THR-ALA ASN

PCU2.1
 ATC ACT ATG CAG ATC TTA AAG ATT CCC CCC AAT GCG GAT CCA CAA TCC TAC GTG GAG ATG
 100
 ILE-THR-MET-GLN-ILE-LEU-LYS-ILE-PRO-PRO-ASN-AAG-ASP-PRO-HIS-SEN-TYR-DNL-GLU-MET-

PCU2.1
 ACC TTC TCT CAG GAT GTA CTC TGC GAA TGC AAG CCT AAT CTG GAG ACC ACC AAG GCA GAA
 120
 THR-PHE-SEN-GLN-ASP-DNL-LEU-CYS-GLU-CYS-AAG-PRO-ILE-LEU-GLU-THR-THR-LYS-ALA-GLU-

AGG TAA
 138
 AAG •

EP 0 476 333 A1



European Patent
Office

EUROPEAN SEARCH REPORT

Application Number

DOCUMENTS CONSIDERED TO BE RELEVANT			EP 91308499.3
Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE CITATION (Int. Cl. 8)
P.X	EP - A - 0 399 816 (MERCK) * Totality *	1-31	C 07 K 15/06 C 07 K 3/28 C 12 N 15/18 C 12 P 21/00
A	WORLD PATENTS INDEX (Online), DERWENT PUBLICATIONS LTD., London Accession No. 90-181 264 * JP-A-2-117 698	1,4,5	A 61 K 37/42
A	WORLD PATENTS INDEX (Online), DERWENT PUBLICATIONS LTD., London Accession No. 89-088 700 * JP-A-1-38 100	1,4,5	
A	EP - A - 0 186 084 (MERCK) * Claims *	1,4,5, 13	
			TECHNICAL FIELDS SEARCHED (Int. Cl. 8)
			C 07 K C 12 N C 12 P A 61 K

The present search report has been drawn up for an inquiry

VIENNA	10-10-1991	MARCHART
CATEGORY OF RELEVANT DOCUMENTS		
<p>1. Documents considered relevant to the invention</p> <p>2. Documents considered relevant to the invention, but not relevant to the claims</p> <p>3. Documents considered relevant to the invention, but not relevant to the claims, and not relevant to the claims</p> <p>4. Documents considered relevant to the invention, but not relevant to the claims, and not relevant to the claims</p> <p>5. Documents considered relevant to the invention, but not relevant to the claims, and not relevant to the claims</p>		